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HEREDITY OF THE Rh BLOOD TYPES

IV. Medicolegal Application in Cases of Disputed Parentage

Alexander S. Wiener, M.D., and Eve B. Sonn
Brooklyn, N. Y.

In A preceding paper[1] data were presented on the Rh blood types in a series of ninety-seven families with 275 children. Only a single contradiction was found to Wiener's theory[2] of six major allelic genes, and in this case the child in question proved to be illegitimate. The original investigation did not yield sufficient data to test the theory adequately with regard to the rarer genes, *Rh'* and *Rh"*, but in a subsequent paper[3] a number of pedigrees were presented which included individuals bearing these rare genes. In the meantime, we have accumulated an additional series of families in which the bloods were tested for the Rh types, and the principal purpose of the present paper is to record these newer results.

In our first study on the heredity of the Rh blood types, we also reported twenty-three medicolegal cases in which tests were carried out for Rh types as well as for the A-B groups, A_1-A_2 subgroups, and M-N types. In the present paper we propose to describe seventy-nine additional cases and to discuss the reliability of the Rh tests when applied in cases of disputed parentage.

MATERIALS AND METHODS

The blood samples tested in the heredity studies were obtained from two main sources: (1) families of acquaintances, technicians, and patients, and (2) families which were investigated because it was suspected that one or more of the children had congenital hemolytic disease (erythroblastosis fetalis). The latter accounts for the relatively large number of families with Rh-negative mothers. The medicolegal cases came from various courts in New York City, especially the Court of Special Sessions, and also included private cases and cases referred by courts outside of New York City.

The technique of performing the grouping, subgrouping, M-N, and Rh tests has been described in detail elsewhere[1, 4, 5] and will not be repeated here. With regard to the nomenclature of the Rh types and Rh antisera, see Wiener.[6, 7]

A number of families was also examined for agglutinogen P. The serum used in these tests came from a patient who had developed anti-P agglutinins as a result of repeated blood transfusions. This case is reported in detail in another paper[8] in which is also described the technique of performing the tests.

RESULTS

In Table I are presented the results of the grouping, subgrouping, M-N, and Rh tests in a series of 91 families with 161 children. In the first 24 families, tests were also carried out for agglutinogen P.

From the Serological Laboratory of the Office of the Chief Medical Examiner of New York City and the Blood Transfusion Division of the Jewish Hospital of Brooklyn.

Aided by grants from the United Hospital Fund of New York City, and from the Carnegie Corporation through the Committee on Human Heredity of the National Research Council.

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TABLE I

LIST OF FAMILY MATERIAL

	FATHER	FAMILY MOTHER	CHILDREN				
1	A_1MP+Rh_1	$A_1MP-Rh-$	A_1MP+Rh_1 ♂	A_1MP+Rh_1 ♂	A_1MP+Rh_1 ♂	A_1MP+Rh_1 ♀	$AMP+Rh_1$ ♂
2	$ONP+Rh_1Rh_2$	$OMP+Rh_1$	$OMNP+Rh_1$ ♂				
3	$ONP+Rh_1$	$ONP+Rh_2$	$ONP+Rh_1Rh_2$ ♂	$ONP+Rh_2$ ♀			
4	$OMNP+Rh_1$	$ONP+Rh-$	$ONP+Rh_1$ ♂	$ONP+Rh_1$ ♀			
5	$A_1MNP-Rh'$	$A_1MP+Rh_1Rh_2$	$OMP-Rh_1Rh_2$				
6	$A_2NP+Rh_1Rh_2$	$ONP+Rh_2$	$ONP+Rh_2$ ♀	$ONP+Rh_1Rh_2$ ♂	$ONP-Rh_1$ ♀	$ONP+Rh_2$ ♀	A_2NP+Rh_2 ♀
			$ONP+Rh_1Rh_2$ ♀	$A_2NP+Rh_1Rh_2$ ♂	$A_2NP-Rh_1Rh_2$ ♀		
7	$OMNP+Rh_1$	A_1NP-Rh_1	$ONP+Rh_1$ ♂	$OMNP+Rh_1$ ♀	$ONP+Rh_1$ ♂	$A_1MNP+Rh_1$ ♀	
8	$OMP+Rh_2$	$OMP+Rh_2$	$OMP+Rh_2$ ♂	$OMP+Rh_2$ ♂	$OMP+Rh_2$ ♂		
9	$ONP-Rh-$	$OMP+Rh-$	$OMNP+Rh-$ ♂	$OMNP+Rh-$ ♀	$OMNP+Rh-$ ♀	$OMNP+Rh-$ ♂	
10	$ONP-Rh_1Rh_2$	$OMNP+Rh_1$	$OMNP-Rh_1$ ♀	$OMNP+Rh_1$ ♂			
11	$A_1MP-Rh_1Rh_2$	$A_1MP-Rh_1Rh_2$	$A_1MP-Rh_1Rh_2$ ♀	$A_1MP-Rh_1Rh_2$ ♂			
12	A_1MP-Rh_1	A_1MP-Rh_2	A_1MP-Rh_1 ♀	$A_1MP-Rh-$ ♀			
13	$OMNP+Rh-$	$OMNP+Rh_1$	$OMNP+Rh_1$ ♀				
14	$ONP+Rh_1Rh_2$	$AMNP-Rh-$	$A_2MNP+Rh_1$ ♂				
15	$A_1MNP+Rh_2$	$A_1MP+Rh-$	A_1MP-Rh_2 ♀				
16	$OMP+Rh_1Rh_2$	$A_1MNP+Rh-$	A_1MP+Rh_2 ♀	A_1MP+Rh_1 ♀	A_1MP+Rh_1 ♀		
17	$OMNP+Rh_1$	$OMNP-Rh-$	$ONP+Rh-$ ♀	$OMP+Rh-$ ♀	$ONP+Rh-$ ♀		
18	$ONP+Rh_1Rh_2$	A_1MP-Rh_1	$A_1MNP-Rh_1$ ♂	$A_1MNP-Rh_1$ ♂	$A_1MNP-Rh_1$ ♂		
19	A_1MP-Rh_1	$A_1MNP+Rh-$	A_1MP+Rh_1 ♀				
20	$ONP+Rh_2$	$OMNP+Rh-$	$ONP+Rh-$ ♂	$OMNP+Rh-$ ♀			
21	$A_1MNP+Rh_1$	$ONP+Rh-$	$ONP+Rh_1$ ♀				
22	$OMNP+Rh_1$	$OMP+Rh_1$	$OMNP+Rh_1$ ♀	$OMNP+Rh_1$ ♀	$OMP+Rh-$ ♂		
23	$OMP+Rh_1$	$OMNP+Rh-$	$OMNP+Rh_1$ ♂	$OMP+Rh-$ ♂			
24	$OMP+Rh_1$	$A_1MNP+Rh_1$	$OMNP+Rh_1$ ♂	$OMP+Rh_1$ ♂	$A_1MNP+Rh_1$ ♂		
25	A_1MNRh-	A_1BMNRh_1	A_1MNRh_1 ♀	A_1BNRh_1 ♂			
26	$BMNRh_2$	$BMNRh-$	$BMNRh-$ ♂	$BMNRh_2$			
27	$A_1MNRh_1Rh_2$	$BMRh_1$	$BMNRh_1$ ♀	A_1MNRh_1 ♀	A_1BMRh_1 ♂	$OMRh_1Rh_2$ ♂	
28	A_2MNRh_2	$OMRh-$	A_2MNRh_2				
29	A_1Rh_1	$ONRh-$	A_1MNRh_1				
30	$A_2BMNRh_1Rh_2$	A_1MNRh-	A_1MRh_2 ♂	A_2NRh_2 ♀			
31	A_1MNRh-	$OMRh_1$	A_1MRh- ♂	$AMNRh-$ ♀			
32	A_1MNRh_1	$A_1BMNRh-$	A_1MNRh_1				
33	$BMRh_1$	A_1MRh-	A_1MRh- ♂	A_1MRh_1 ♂			
34	$ONRh_1$	$OMNRh-$	$OMNRh_1$				
35	$OMNRh_1$	A_1MRh-	$OMNRh_1$ ♀	$AMNRh_1$			
36	$BMRh_1$	$OMNRh_1$	$BMRh_1$ ♀				
37	$ONRh_2$	$BMNRh_1$	$OMNRh'$				
38	$OMNRh_1$	A_1MNRh-	$OMNRh_1$				
39	$ONRh_1Rh_2$	A_1MNRh-	$AMNRh_2$ ♂	$AMNRh_1$			
40	$OMNRh-$	$OMNRh-$	$ONRh-$ ♀				
41	$BNRh-$	$BMNRh_1Rh_2$	$OMNRh_1$ ♂				
42	$BMNRh_1$	$OMRh-$	$OMNRh-$				
43	$OMRh_1$	$BNRh-$	$OMNRh_1$				
44	A_1BMNRh_1	A_1MNRh-	A_1NRh_1 ♂				
45	A_1MRh_2	A_1MRh-	A_1MRh_2 ♂	A_1MRh_2			
46	$A_1MRh_1Rh_2$	$OMNRh-$	$AMRh-$				
47	A_1MNRh_1	A_1MNRh-	$OMNRh_1$ ♀	A_1MRh_1 ♀			

43	$OMRh_1$	$BNRh-$	$OMNRh_1$			
44	A_1BMNRh_1	A_1MNRh-	$A_1NRh_1♂$			
45	A_1MRh_2	A_1MRh-	$A_1MRh_2♂$	A_1MRh_2		
46	$A_1MRh_1Rh_2$	$OMNRh-$	$AMRh-$			
47	A_1MNRh_1	A_1MNRh-	$OMNRh_1♀$	$A_1MRh_1♀$		
48	$A_1MNRh_1Rh_2$	$BMNRh_1$	A_1BNRh_1			
49*	A_1MNRh-	$A_{1,2}MNRh_o$	$AMNRh_o$			
50	A_1MNRh_2	$OMRh-$	$OMNRh_2$	$OMRh_2$		
51	A_1MNRh_1	$ONRh_1$	A_1MNRh_1			
52	$A_1BMRh_1Rh_2$	$BMRh-$	A_1BMRh_2			
53	$AMRh-$	$BNRh_1$	$AMNRh-$			
54	$BMNRh_1$	A_1MRh-	$AMNRh_1♂$			
55	$A_1MRh_1Rh_2$	$ONRh_o$	$AMNRh_1♂$			
56	A_1MRh_2	A_1MRh_1	$A_1MRh_1Rh_2♂$			
57	$ONRh_1Rh_2$	$A_1BMNRh_1Rh_2$	$BMNRh_1Rh_2♂$			
58	$ANRh_1$	$OMNRh_1$	$OMNRh_1♀$			
59	A_1MNRh_1	$OMRh'$	$A_1MRh_1♂$			
60	$A_1MNRh_1Rh_2$	$BMNRh-$	$A_1BMRh_1♂$			
61	$OMRh_1Rh_2$	$A_1BMNRh-$	$A_1MNRh_1♂$	$A_1MNRh_1♀$		
62	$OMRh_1$	$ONRh-$	$OMNRh_1$			
63	A_1MRh_1	A_1MRh_o	$A_1MRh-♂$			
64*	A_1MNRh-	$A_{1,2}MNRh_o$	A_2MNRh_o			
65	$OMNRh_1Rh_2$	$BMNRh-$	$BMNRh_1$			
66	$BMNRh_1$	A_1MRh-	$A_1BMRh_1♂$	$ABMNRh_1$		
67	$OMNRh_1Rh_2$	A_1MNRh-	$A_1NRh_1♀$	$OMNRh_2♀$		
68	$ONRh_1$	A_2MRh-	$OMNRh_1♀$	$OMNRh_1♀$		
69	$OMNRh_1$	A_1MNRh-	$OMNRh_o♂$			
70	$A_1BMRh_1Rh_2$	$OMNRh_1Rh_2$	$A_1MNRh_1♂$			
71	$BMNRh_1$	A_1NRh-	A_1BNRh_1			
72	A_2MNRh_1	$OMNRh-$	$A_2NRh-♂$	$A_2MNRh_1♀$		
73	$ONRh_1$	$OMNRh-$	$ONRh-♀$	$ONRh-$		
74	$OMRh_2$	A_1MRh-	$A_1MRh_2♂$	$OMRh_2♂$		
75	$A_1BMNRh_1Rh_2$	$OMRh_1Rh_2$	$AMNRh_2$			
76	$OMNRh_1$	$OMNRh_2$	$ONRh_1Rh_2♂$	$ONRh_1♀$	$OMRh_1Rh_2♂$	
77	$BMRh-$	A_1NRh_1	$A_1BMNRh_1♂$ †	$A_1BMNRh_1♂$ †	$OMNRh_1♂$ †	
78	$OMNRh'$	$OMNRh_1$	$ONRh_1♂$			
79	A_1NRh_2	A_2MRh_1	$A_1MNRh_1♂$	$A_2MNRh_1Rh_2♀$ ‡	$A_2MNRh_1Rh_2♀$ ‡	
80	$OMRh_1$	$OMRh-$	$OMRh_1♂$			
81	$OMNRh_1$	A_1MNRh_1	$OMNRh_1♂$			
82	$OMRh_1$	$OMNRh''$	$OMNRh_1Rh_2$			
83	A_2MNRh_1	$BMRh-$	$BMNRh_1♂$	$OMNRh_1♂$		
84	A_1NRh_1	A_2NRh-	A_1NRh_o			
85	A_1MRh_1	$OMNRh_1$	A_1MNRh_1			
86	A_2MRh_2	A_1MNRh-	$A_1MRh-♀$			
87	A_1NRh_1	$OMRh_1$	$OMNRh_1$			
88	$OMNRh_1$	$OMNRh_1$	$ONRh_1♀$			
89	$OMNRh_1$	$OMNRh_2$	$OMNRh-♀$			
90	A_2MRh-	$BNRh_2$	$A_2BMNRh_2♂$			
91	$OMNRh_2$	$ONRh-$	$OMNRh_2♂$	$OMNRh_2♂$	$ONRh-♂$	$ONRh_2♀$
			$OMNRh-♂$	$ONRh-♂$	$ONRh-♂$	

*Negro families. †Triplets. ‡Newborn twins.

In Table II, the hereditary transmission of the Rh types in the ninety-one families is summarized.* With only a single apparent exception (Family 46), the results conform with the theory that the eight Rh types are transmitted by a series of six allelic genes, *Rh*$_1$ (or *Rh*$_o'$), *Rh*$_2$ (or *Rh*$_o''$), *Rh'*, *Rh''*, *Rh*$_o$, and *rh*. In view of the importance of the apparent exception to the theory, this case will be described in detail.

TABLE II

SUMMARY OF FAMILY MATERIAL OF TABLE I

MATING	NUMBER OF FAMILIES	NUMBER OF CHILDREN OF TYPES						
		Rh−	Rh_1	Rh_2	Rh_1Rh_2	Rh_o	Rh′	TOTALS
Neg. × Neg.	2	5	0	0	0	0	0	5
Neg. × Rh_1	32	12	36	0	0	4	0	52
Neg. × Rh_2	10	9	0	13	0	0	0	22
Neg. × Rh_1Rh_2	11	(1)	10	6	0	0	0	17
Neg. × Rh_o	2	0	0	0	0	2	0	2
Rh_1 × Rh_1	10	1	16	0	0	0	0	17
Rh_1 × Rh_2	7	2	3	1	6	0	1	13
Rh_1 × Rh_1Rh_2	5	0	10	0	1	0	0	11
Rh_1 × Rh_o	1	1	0	0	0	0	0	1
Rh_1 × Rh′	2	0	2	0	0	0	0	2
Rh_1 × Rh″	1	0	0	0	1	0	0	1
Rh_2 × Rh_2	1	0	0	3	0	0	0	3
Rh_2 × Rh_1Rh_2	1	0	1	3	4	0	0	8
Rh_1Rh_2 × Rh_1Rh_2	4	0	1	1	3	0	0	5
Rh_1Rh_2 × Rh_o	1	0	1	0	0	0	0	1
Rh_1Rh_2 × Rh′	1	0	0	0	1	0	0	1
Totals	91	31	80	27	16	6	1	161

The mother of Family 46 was admitted in labor to the obstetric ward of Bellevue Hospital with the following history. Her first pregnancy had resulted in a normal male child who was 6 years old and well, while the second had resulted in a normal female child who was 4 years old and well. Neither of these two children was available for examination. The patient's third pregnancy had terminated two and one-half years ago with a stillborn male infant. At that time the woman was found to be Rh negative with strong anti-Rh agglutinins in her serum, indicating that the stillbirth was a manifestation of hemolytic disease. The present pregnancy was her fourth one. Tests were carried out on patient and husband and it was found that the husband belonged to type Rh_1Rh_2. It therefore appeared certain that the expected infant would have hemolytic disease because it had to be Rh positive. Much to everyone's surprise, the newborn infant proved to be normal in every respect. Serologic tests then showed the child to be Rh negative which explained why it had escaped the disease, but this raised a new problem because this finding was contrary to the expectations under the genetic theory. When the patient was confronted with these facts she practically admitted that her husband was not the father of the infant.

No contradictions were encountered to the laws of inheritance of the blood groups, subgroups or M-N types in any of the families. Inasmuch as only a few studies have been carried out to date on the heredity of the agglutinogen P, our results in twenty-four families have been summarized in Table III. It will be seen that our findings conform with the theory that the agglutinogen P is transmitted as a simple Mendelian dominant.[10]

In Table IV, we have summarized the data from our pedigrees illustrating the transmission of the rare gene *Rh'* and *Rh,"* which were reported in a previous paper as already mentioned. In Table V we have combined the data of Tables II and IV with the family data presented in our first genetic study of the Rh blood types. It will be seen that to date we have determined the

*In two families (2 and 18) there were individuals with blood giving intermediate reactions.[9] For the sake of simplicity, data on these individuals are included in Table II together with the data on the type Rh_1 individuals.

TABLE III

HEREDITY OF AGGLUTINOGEN P IN FAMILIES OF TABLE I

MATING	NUMBER OF FAMILIES	CHILDREN		
		P+	P–	TOTALS
P+ × P+	13	29	3	32
P+ × P–	9	19	5	24
P– × P–	2	0	4	4
Totals	24	48	12	60

TABLE IV

SUMMARY OF FAMILY MATERIAL FROM STUDY OF SONN AND WIENER

MATING	NUMBER OF FAMILIES	NUMBER OF CHILDREN OF TYPES							
		Rh–	Rh_1	Rh_2	Rh_1Rh_2	Rh_0	Rh′	Rh″	TOTALS
Neg. × Rh′	1	1	0	0	0	0	1	0	2
Neg. × Rh″	1	1	0	0	0	0	0	5	6
Rh_1 × Rh_2	1	0	1	0	0	0	0	0	1
Rh_1 × Rh_1Rh_2	1	0	6	2	2	0	0	0	10
Rh_1 × Rh′	1	0	1	0	0	0	0	0	1
Rh_1 × Rh″	3	1	2	0	2	0	0	0	5
Rh_0 × Rh′	1	0	0	0	0	1	1	0	2
Totals	9	3	10	2	4	1	2	5	27

TABLE V

SUMMARY OF AUTHORS' FAMILY MATERIAL TO DATE

MATING	NUMBER OF FAMILIES	NUMBER OF CHILDREN OF TYPES							
		Rh–	Rh_1	Rh_2	Rh_1Rh_2	Rh_0	Rh′	Rh″	TOTALS
Neg. × Neg.	4	14	0	0	0	0	0	0	14
Neg. × Rh_1	49	25	73	0	0	7	0	0	105
Neg. × Rh_2	16	10	0	20	0	0	0	0	30
Neg. × Rh_1Rh_2	15	(1)	18	14	0	0	0	0	33
Neg. × Rh_0	3	3	0	0	0	3	0	0	6
Neg. × Rh′	2	1	0	(1)	0	0	1	0	3
Neg. × Rh″	1	1	0	0	0	0	0	5	6
Rh_1 × Rh_1	26	5	62	0	0	4	1	0	72
Rh_1 × Rh_2	21	6	15	7	18	0	2	0	48
Rh_1 × Rh_1Rh_2	27	0	46	8	25	0	0	0	79
Rh_1 × Rh_0	1	1	0	0	0	0	0	0	1
Rh_1 × Rh′	3	0	3	0	0	0	0	0	3
Rh_1 × Rh″	4	1	2	0	3	0	0	0	6
Rh_2 × Rh_2	2	1	0	4	0	0	0	0	5
Rh_2 × Rh_1Rh_2	6	0	2	8	8	0	0	0	18
Rh_2 × Rh_0	2	0	0	1	0	4	0	0	5
Rh_1Rh_2 × Rh_1Rh_2	10	0	3	1	17	0	0	0	21
Rh_1Rh_2 × Rh_0	2	0	1	1	0	0	0	0	2
Rh_1Rh_2 × Rh′	2	0	1	1	2	0	0	0	4
Rh_0 × Rh′	1	0	0	0	0	1	1	0	2
Totals	197	69	226	66	73	19	5	5	463

Rh blood types in 197 families with 463 children. In the entire series, there were only two apparent exceptions to the theory of six allelic genes (indicated by the parentheses in Table V), both of which have been readily explained because the children in question were shown to be illegitimate.

MEDICOLEGAL APPLICATIONS

Our family studies on the Rh blood types as well as statistical studies on the distribution of the types in the general population[11] have proved the accuracy of the theory of six allelic genes beyond any reasonable doubt. Moreover,

TABLE VI

RESULTS OF TESTS IN CASES OF DISPUTED PARENTAGE

CASE	PUTATIVE FATHER	MOTHER	CHILDREN	INTERPRETATION
1*	A_1MNRh_o	A_2NRh_o	A_1MNRh_o ♂	No exclusion
2	$OMRh_2$	$OMNRh'$	$OMNRh-$ ♂	No exclusion
3	A_1MNRh_1		A_2MNRh_1 ♀	No exclusion
4	$ONRh_1Rh_2$	$OMRh_1$	a) $OMNRh_1$ ♂ b) $OMNRh_1Rh_2$ ♂ c) $OMNRh_1Rh_2$ ♂	None of the children excluded
5*	A_2NRh_o	$OMNRh_2$	A_2MNRh_2 ♂	No exclusion
6	$A_1BMNRh_1Rh_2$	$OMNRh_1$	$BMRh_1Rh_2$ ♀	No exclusion
7	A_2MNRh_1	$BMNRh_2$	A_2BMNRh_2 ♀	No exclusion
8	A_1NRh_2	A_1NRh_1	$OMNRh_1$ ♀	Excluded by M-N tests
9†	$BMNRh_2$	$BMNRh-$	$BMRh_o$	No exclusion
10	A_1BMNRh_1	$OMNRh_1$	a) $BMNRh_1$ ♀ b) $BMNRh_1$ ♂	Neither child excluded
11	A_1MNRh_1	A_1MNRh_1	A_1MNRh_1 ♀	No exclusion
12	$OMRh_1$	$BNRh_1Rh_2$	A_1MNRh_2 ♂	Exclusion by A-B-O tests
13	$OMNRh_1$	$OMNRh_1$	$OMNRh_1$ ♂	No exclusion
14	a) $OMRh_1$ b) A_1MNRh_1	$OMRh_1$	$OMRh_1$ ♀	Neither man excluded
15	A_1MNRh_1	$A_1MNRh_1Rh_2$	$A_1NRh_1Rh_2$ ♂	No exclusion
16	$BMRh_1$	$OMRh_1$	$OMRh_1$ ♀	No exclusion
17	A_1MRh_1	A_2BMNRh_1	a) $BMRh_1$ ♂ b) $BNRh_1$ ♂	Second child excluded by M-N tests
18	$A_1NRh_1Rh_2$	$OMNRh_1$	$OMRh_1$ ♂	Excluded by M-N tests
19	$BMNRh_1$	$OMNRh-$	$BMNRh_1$ ♂	No exclusion
20*	$OMNRh_o$	A_2NRh_2	A_2MNRh_o ♀	No exclusion
21	$OMRh_1Rh_2$	$BMNRh_1$	$OMNRh_1Rh_2$ ♀	No exclusion
22	$BNRh-$	$OMRh_1$	$OMNRh-$ ♂	No exclusion
23†	$OMNRh_1$	$BMRh_1$	$BMRh_1$ ♂	No exclusion
24†	$BMNRh_1$	$ONRh-$	a) $BMNRh_1$ ♀ b) $BMNRh-$ ♂	Neither child excluded
25	$A_{1,2}MNRh_1$	$OMNRh-$	A_1MRh_1 ♂	No exclusion
26*	$OMRh_o$	A_2NRh_o	A_1MNRh_o ♂	No exclusion
27‡	A_1BMNRh_1	$BNRh_o$	$BNRh-$ ♂	No exclusion
28	$OMRh_1$	A_2MNRh_1	A_2MNRh_1 ♀	No exclusion
29	A_2MNRh_1	$A_2NRh_1Rh_2$	A_2MNRh_2 ♂	No exclusion
30	A_1BNRh_1	A_1MRh_1	A_1MNRh_1 ♀	No exclusion
31†	A_1MRh_2	$BNRh_1$	A_1MNRh_1 ♂	No exclusion
32†	$OMRh_1Rh_2$	A_2MRh_2	A_1MRh_2 ♀	Exclusion by A_1-A_2
33*	$A_2NRh_1Rh_2$		A_1BMRh_o ♂	Triple exclusion by A_1-A_2, M-N, and Rh tests
34	$ONRh_1$	$A_{1,2}MRh_2$	$A_{1,2}MRh-$ ♂	Exclusion by M-N tests
35	A_2MNRh_1	$OMRh_1$	a) $OMNRh_1$ ♀ b) $OMNRh-$ ♂	Neither child excluded
36*	$OMRh_1$	$BMNRh_1$	$BMRh_1$ ♀	No exclusion
37	$OMNRh_1$	A_1MRh_1	$OMRh_1$ ♂	No exclusion
38	$ONRh-$	$BMNRh_1$	$BNRh_1$ ♀	No exclusion
39	A_1NRh-	A_1MNRh_1	A_1NRh_1 ♂	No exclusion
40†	A_1MRh_2	$OMNRh_1$	$OMRh_1$	No exclusion
41†	A_1MNRh_1	A_2MNRh_1	A_2NRh_1	No exclusion
42	A_2MNRh_1	A_1MNRh_1	$A_1MNRh_1Rh_2$ ♀	Exclusion by Rh tests
43	$A_1MRh_1Rh_2$	$OMRh_1$	A_1MRh_1 ♂	No exclusion
44	$OMNRh_1$	A_1MRh-	A_1MRh_1 ♀	No exclusion
45	A_1MRh_2	A_1MRh_1	$A_1MRh_1Rh_2$ ♀	No exclusion
46	$OMNRh_1Rh_2$	$OMNRh_1$	$OMNRh_2$ ♀	No exclusion
47*	A_1NRh-	$ONRh_o$	$ONRh_o$ ♂	No exclusion
48	$OMRh_2$	$BMNRh_1Rh_2$	$BMRh_1Rh_2$ ♀	No exclusion
49	$OMNRh_2$	$OMNRh-$	$OMNRh-$ ♀	No exclusion

*Negro.
†Race not known; blood samples received by mail.
‡Man, Chinese; woman, Negro.
§Man, Negro; woman, white.
‖M-N tests on baby not reliable because of blood transfusions.
¶Man, American Indian; woman, Negro.
**Twins.

TABLE VI—CONT'D

CASE	PUTATIVE FATHER	MOTHER	CHILDREN	INTERPRETATION
50	$BMRh_1$	$OMRh_2$	$BMRh_1$ ♀	No exclusion
51	$OMNRh_2$	$OMNRh_2$	$ONRh_-$ ♂	No exclusion
52§	$A_2MNRh_1Rh_2$	$OMNRh_1$	$OMNRh_2$ ♀	No exclusion
53‖	$A_1MNRh_1Rh_2$	$OMNRh_1Rh_2$	ORh_1Rh_2 ♂	No exclusion
54*	A_1MNRh_2	$BMNRh_2$	A_2BMNRh_2 ♂	No exclusion
55	A_1NRh-	$OMRh_1$	$OMNRh_1$ ♀	No exclusion
56	$OMNRh-$	A_2NRh_1	$ONRh_1$ ♂	No exclusion
57	$OMNRh_1$	$ONRh-$	$OMNRh_1$ ♂	No exclusion
58	$OMNRh_1$	$OMNRh-$	$OMNRh_1$ ♂	No exclusion
59*	$OMNRh_1$	$A_1MNRh_1Rh_2$	A_1MRh_1 ♀	No exclusion
60	A_2BMNRh_1		A_2BNRh_1 ♀	No exclusion
61	A_1MRh_1	A_1MNRh_1	A_1MRh_1 ♀	No exclusion
62*	$BMRh_2$	A_1NRh_0	A_1MNRh_0 ♂	No exclusion
63*	A_1NRh_1	A_2MRh_1	$A_2MRh_1Rh_2$ ♂	Double exclusion by M-N and Rh tests
64	A_1MRh_1	$A_2MNRh_1Rh_2$	$OMNRh_1$ ♂	No exclusion
65*	$OMNRh-$	$OMNRh_0$	a) $OMNRh_0$ ♀ b) $OMNRh_0$	Neither child excluded
66	$BMRh_1$	$OMRh_1$	$BMRh_1$ ♀	No exclusion
67¶	$OMRh_2$	$OMNRh_0$	$BMNRh_0$ ♂	Exclusion by A-B-O tests
68†	A_1MNRh_1	A_1MRh_1	A_1MNRh_1	No exclusion
69	$BNRh_1$	$ONRh_-$	$BNRh_1Rh_2$ ♀	No exclusion
70	$OMNRh_1$	$A_1MRh_1Rh_2$	$A_1MNRh_1Rh_2$ ♀	No exclusion
71	$BMNRh_1$	A_1MRh_1	$BMRh_1$ ♂	No exclusion
72	$OMNRh_1Rh_2$	$OMNRh_1Rh_2$	A_2NRh_1 ♂	Exclusion by A-B-O tests
73†	$OMNRh_2$	$OMRh_2$	$OMNRh_2$ ♂	No exclusion
74	$BMNRh_1Rh_2$	A_1MNRh_1	$A_1BMNRh_1Rh_2$ ♂	No exclusion
75*	A_2NRh_2	$OMNRh_1$	a) $ONRh_0$ ♂ b) A_2MNRh_0 ♀	Neither child excluded
76	$OMNRh_1$	$OMNRh_1$	$OMNRh_1$ ♀	No exclusion
77°	$BMNRh_1Rh_2$	A_2BMNRh_1	$BMNRh_1$ ♀	No exclusion
78°	$ONRh_1$	$BMNRh_0$	a) A_2BMNRh_0 ♂ ** b) $BMRh_0$ ♂ **	First child excluded by A-B tests; second child excluded by M-N tests
79	A_1MNRh-	A_1NRh_-	A_1MNRh'' ♂	No exclusion

Race and co-workers,[12] working independently in England, have collected data in a series of fifty-six families with ninety children, all conforming with the expectations under the theory. Therefore, the medicolegal application of the Rh blood types in cases of disputed parentage is justified and, as has already been mentioned, for the past eighteen months we have carried out Rh typings routinely together with the grouping, subgrouping, and M-N tests in all cases of disputed parentage. In Table VI, seventy-nine new cases of disputed paternity are listed which, together with our previously reported series of twenty-three, bring our total beyond the 100 mark.

The application of the Rh types for the exclusion of paternity is based on the following laws of inheritance:

1. The factors Rh_0 and Rh′ and Rh″ are transmitted as simple Mendelian dominants and therefore cannot appear in the blood of a child unless present in the blood of one or both parents. In applying this law it must be borne in mind that while there are three Rh factors, there are five agglutinogens, Rh_0, Rh′, Rh_1, Rh″, and Rh_2, where Rh_1 contains the factors Rh_0 and Rh′ together and Rh_2 contains the factors Rh_0 and Rh″ together. While agglutinogens Rh_1 and Rh_2 are usually transmitted as units by the corresponding genes Rh_1 and Rh_2, in occasional individuals (genotype Rh_0Rh' and Rh_0Rh'') the factors will exist as distinct agglutinogens that segregate genetically. Therefore, while agglutinogens Rh_1 and Rh_2 will usually behave as if they were simple Mendelian dominants, there will be rare families where the child will belong to type Rh_1 (or type Rh_2) and yet neither parent will have the agglutinogen Rh_1

(or Rh_2). For example, in the rare mating $Rh_0 \times Rh'$, usually one-fourth of the children will give reactions corresponding to type Rh_1.

2. Parents belonging to type Rh_1Rh_2 or the rare type Rh'Rh" cannot have children belonging to type Rh_0 or Rh negative. Similarly, parents of types Rh_0 and Rh negative cannot have children of type Rh_1Rh_2 or Rh'Rh". This law is obvious because parents of types Rh_1Rh_2 and Rh'Rh" must transmit one of the genes Rh_1, Rh_2, Rh', or $Rh"$ to each child, while parents of types Rh_0 and Rh negative can transmit only either an Rh_0 or rh gene to each child.

3. Further exclusions of parentage are possible when more than one child is available for testing. For example, in the mating $Rh_1 \times$ Rh negative, if there is one Rh-negative child, then the Rh_1 parent must belong to genotype Rh_1rh, and children belonging to any type other than Rh_1 or Rh negative will be excluded. On the other hand, in the mating $Rh_1 \times$ Rh negative, if there are any children of types Rh_0 or Rh', there can be no Rh-negative children. This law will rarely be applicable in medicolegal cases because these usually involve only a single child.

The value of the Rh tests in cases of disputed paternity can be gleaned from the fact that whereas previously, with the aid of the A-B-O and M-N tests, a falsely accused man had one chance in three of proving his innocence, the Rh tests have raised the chances of exclusion to approximately 45 per cent. In the present series, the accused man was excluded by the Rh tests in three cases. In one case (63) the man was also excluded by the M-N tests. The second case (33) is of interest because even though the mother's blood was not available for testing, the accused individual, a young Negro lad, was excluded by three separate tests. He belonged to subgroup A_2 and the child to subgroup A_1B; he belonged to type N and the child to type M; and he belonged to type Rh_1Rh_2 and the child to type Rh_0. In the third case (42), the man was excluded by the Rh tests where the other tests were inconclusive. Together with our previously reported exclusion (Case 3, Wiener, Sonn, and Belkin[1]) there were two cases among our total of 102 cases, in which the man was excluded by the Rh tests, but the woman's false accusation might have been believed if we had had to depend only on the A-B-O and M-N tests.

It may be of some interest to point out that there was one exclusion involving the subgroups of A (Case 32). In another interesting case (25) the blood of the putative father, who belonged to group A, gave intermediate reactions with the absorbed B serum; the mother belonged to group O and the child to subgroup A_1. The difference in reactivity between the blood of the putative father and child with the absorbed B serum was not considered evidence of nonpaternity because of the limited knowledge concerning the hereditary transmission of the intermediate subgroups of A.

OTHER APPLICATIONS OF THE Rh BLOOD TYPES

The Rh blood types are also useful for the individual identification of fresh, wet bloodstains. The common blood groups and subgroups give rise to six types of blood, the agglutinogens M and N determine three types of blood, with anti-P serum two types of blood can be distinguished, while with the three varieties of anti-Rh sera and with anti-Hr serum,[5, 13] eight types of blood can be distinguished (disregarding the rare types Rh'Rh" and homozygous Rh'), so that in all $6 \times 3 \times 2 \times 8 = 288$ varieties of human blood can readily be differentiated.

In a previous paper[14] it was shown that the Rh genes are not sex-linked and most likely are carried in a different pair of chromosomes from the *A-B-O* and *M-N* genes. The discovery of the existence of six major allelic genes in the Rh series has materially increased the value of these tests for linkage studies in human genetics.

A third application is in relation to the problem of superfecundation. If an Rh-negative mother gave birth to triplets each belonging to a different Rh type, this could only be explained by assuming that at least two of the triplets had different fathers.

COMMENT

In this paper, for the sake of simplicity, we have confined the presentation to the six standard genes and the eight Rh types which they determine. No attempt has been made to discuss the Hr factor or Race and Taylor's[15] rare Rh_y and Rh_z genes or the rare intermediate genes described by Wiener.[8] The existence of these rare genes complicates the heredity mechanism, making it necessary to apply the second law of inheritance with some reservation, although no qualification is necessary for exclusions of paternity based on the first law.

With regard to the nomenclature of the Rh genes and types, at first a numbered system was used by us, but the present nomenclature was substituted as soon as the serology and genetics were worked out. Murray's[16] contention that the discovery by Race and Taylor of their Rh_y gene makes necessary a total revision of the nomenclature is fallacious, because the present designations can easily be extended to include any additional genes that may possibly be encountered (cf. Wiener[9]). Incidentally, there was no need to change the names of the groups and subgroups or the M-N types when the rare genes A_3 and N_2 were discovered. Aside from the fact that Murray's designations do not take cognizance of the special serologic and genetic positions of the anti-Rh_0 and anti-Hr sera in relation to anti-Rh′ and anti-Rh″, they introduced two hypothetical sera which most likely do not even exist. Finally, it hardly seems a simplication to call blood of type Rh_1Rh_2 "135-123," while Rh-negative blood is designated by Murray as "456."

SUMMARY

Data have been presented on the Rh types in a series of ninety-one families with 161 children. A single apparent exception to the theory of six allelic genes has been shown to be due to illegitimacy. These data combined with previously published results make a total of 197 families with 463 children tested by us for the Rh blood types. The satisfactory agreement between the observations and expectations under the theory justify the medicolegal application of the Rh tests for the exclusion of paternity.

To date, in 102 cases of disputed paternity, tests for the Rh types have been carried out together with the usual A-B-O, A_1-A_2, and M-N tests. In four cases the accused man was excluded by the Rh tests; in two of these cases he was also excluded by one of the other tests, but in two cases the woman's false accusation might have been believed had the Rh tests not been performed.

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THE RELATIONSHIP BETWEEN CHANGES IN SERUM PROTEIN CONCENTRATION AND THE PLASMA VOLUME IN NORMAL SUBJECTS

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INTRODUCTION

CHANGES in the concentration of the serum proteins have been used as an index of alterations in the volume of the plasma. This is based on the concept that, in the absence of capillary damage, the amount of protein in the blood stream remains constant. The demonstration by Madden and Whipple,[1] that serum protein is in a state of "dynamic equilibrium" and may be added to or taken from the blood stream with ease, would suggest that the plasma protein concentration would not necessarily be a good index of increases or decreases in the plasma volume.

This is a report of a comparison between induced changes in the plasma volume and the accompanying variations in the total protein concentration and the total circulating protein in normal subjects. It indicates that the changes in serum protein concentration fail to reflect proportionate alterations in the plasma volume because, under these circumstances, serum protein may be added to or taken from the blood stream.

METHODS AND MATERIAL

The subjects were normal medical students or ambulatory hospital patients, in good health at the time of the observations, who had been on a routine hospital diet for some time. As far as could be determined at the time of the studies, they were in a good state of nutrition and normal hydration. None had had previous edema or cardiovascular or renal disease.

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Increases in the plasma volume greater than 5 per cent were produced in forty-four instances by the daily oral administration of 20 Gm. of sodium bicarbonate, or 25 Gm. of sodium chloride,[2] the administration of desoxycorticosterone acetate intramuscularly,[3] or the ingestion of large amounts of water. Decreases in the plasma volume greater than 5 per cent were produced on forty-one occasions by the administration of ammonium chloride,[4] mercupurin,[5] or both, and by simple dehydration.

Plasma volume determinations were made by the method of Gibson and Evelyn.[6] Fifteen to twenty-five minutes were allowed for the mixing of the dye and subsequently six samples were taken under oil at five- to six-minute intervals. The serum protein concentration was determined by the falling drop method of Barbour and Hamilton[7] on the serum drawn for the plasma volume.

All plasma volume determinations were made in the morning with the patient supine in the rested postabsorptive state. Following the initial determination, the subject was placed on a regime affecting the water and salt content of the body and the determinations repeated twenty-four, forty-eight, or seventy-two hours later under the same conditions. The subjects receiving only large amounts of water remained in the rested fasting state but had the determinations repeated in from three to five hours.

From the product of the plasma volume and the concentration of serum protein per 100 c.c. of serum, the total amount of protein present in the serum may be calculated. Since the plasma volume varies with the size of the individual, the extent of alterations in the plasma volume, serum protein concentration, and total circulating protein are for the purpose of comparison expressed as percentage change from the initial determination. The relationship between alterations in these three factors has been studied by a comparison of the mean percentage change with increases and decreases in the plasma volume, the coefficient of correlation, and by a comparison of the individual changes to the standard error of estimate from a linear regression for increased and decreased plasma volume and for the two groups combined.

The determinations of the plasma volume, serum protein concentration, and total circulating protein remain quite constant from day to day in normal subjects following their usual daily routine. In Table I is shown the reliability of the determinations in control subjects on whom observations were repeated on successive mornings.

TABLE I

CHANGES FOUND IN CONTROL SUBJECTS WITH DETERMINATIONS MADE ON SUCCESSIVE MORNINGS

	NUMBER OF PAIRED OBSERVATIONS	MEAN PERCENTAGE CHANGE (PER CENT)	STANDARD DEVIATION OF MEAN (PER CENT)	STANDARD ERROR OF MEAN	PROBABLE INCIDENCE OF CHANGES GREATER THAN +5 PER CENT	PROBABLE INCIDENCE OF CHANGES GREATER THAN −5 PER CENT
Plasma volume	22	+0.68	2.80	0.61	0.07	0.02
Total serum protein concentration	13	−0.60	3.49	1.0	0.06	0.11
Total circulating protein	13	+0.29	3.71	1.07	0.10	0.08
Hematocrit	22	−0.79	2.63	0.56	0.02	0.06
Red blood cell volume	22	−2.32	4.78	1.04	0.06	0.30

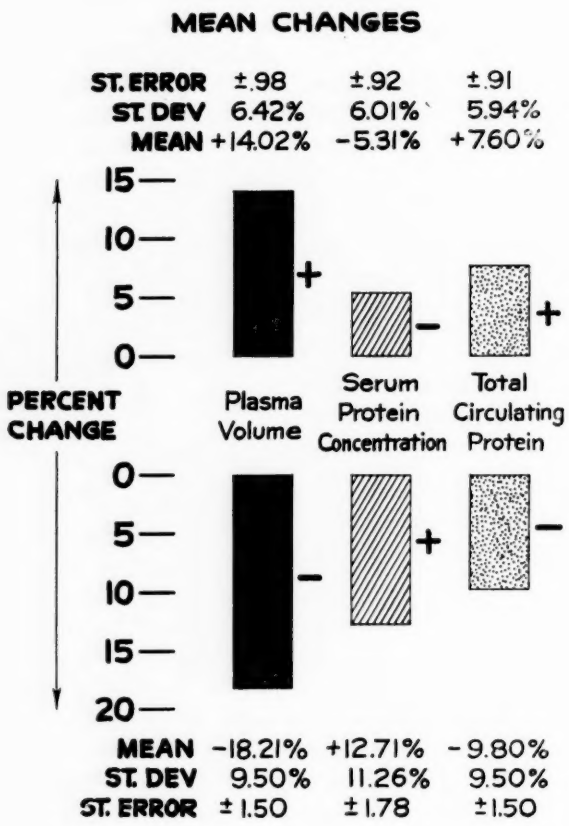

Fig. 1.—Mean percentage change in plasma volume, serum protein concentration, and total circulating protein in normal subjects with induced increases and decreases in plasma volume.

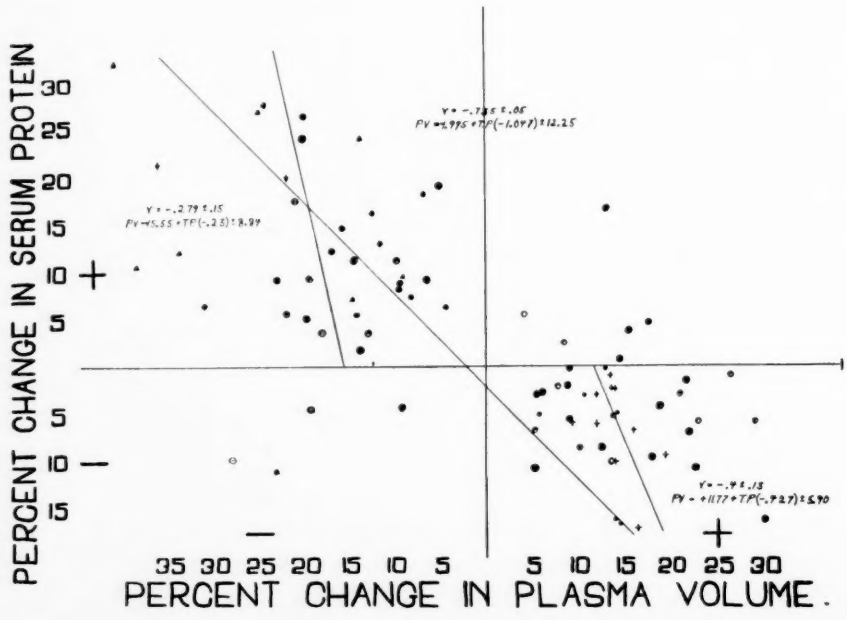

Fig. 2.—Individual variations in the percentage change of serum protein concentration and plasma volume and their relation to a linear regression.

RESULTS

The mean percentage change in the plasma volume, serum protein concentration, and total circulating protein for each group of observations are shown in Fig. 1. The total circulating protein was altered in the direction of the shift in plasma volume. The mean percentage change in total circulating protein represented about 54 per cent of the mean percentage variation in the plasma volume in both groups. The serum protein concentration failed to reflect the extent of the alteration in the plasma volume. This was especially true with increases in the plasma volume where the mean percentage change in serum protein concentration represented only 37.8 per cent of the mean alteration in the plasma volume.

The variation in the relative changes in the plasma volume and serum protein concentration may be better visualized by a graph of the individual observations (Fig. 2). It will be noted that with decreases of plasma volume, the serum protein concentration changed considerably, with large inconsistent variations. Increases in the plasma volume, on the other hand, were associated with small decreases in the concentration of serum protein which, in all but four of the subjects, changed less than 10 per cent. It is apparent from an inspection of this graph that a linear relationship between percentage change in plasma volume and serum protein concentration for both groups may be described only with considerable error.

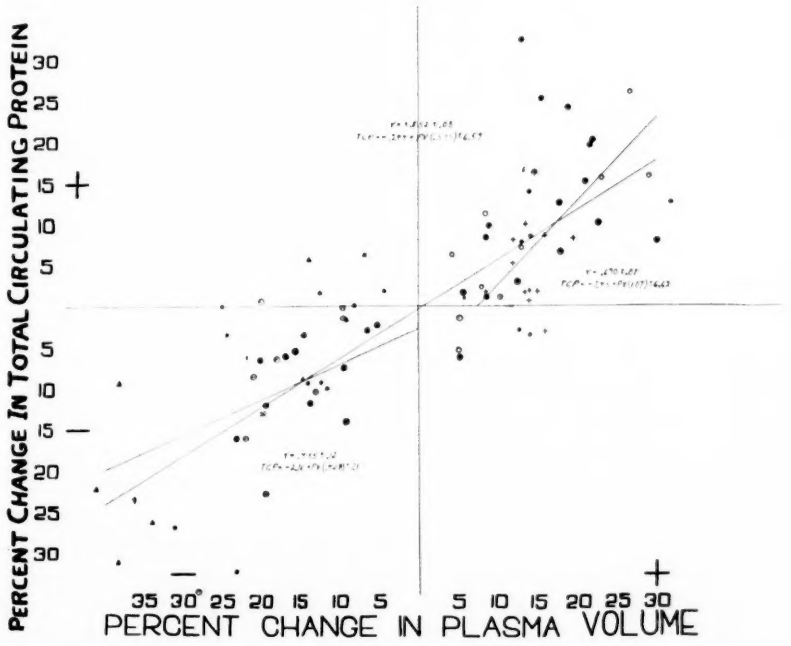

Fig. 3.—Individual variations in plasma volume and total circulating protein and their relation to a linear regression.

The coefficient of correlation between the relative variation in plasma volume and serum protein concentration was satisfactory when both groups were combined but of no significance in either group alone, indicating that there appears to be only a directional change in serum protein concentration with change in plasma volume. A prediction of the change in plasma volume directly from change in serum protein concentration would not only be subject to considerable

error, but also tend to underestimate the plasma volume, especially when the serum protein concentration is decreased. Prediction of the plasma volume from the change in serum protein concentration with the linear regression formula in this data has an error of estimate of 12.3 per cent.

The relationship between the variation in plasma volume and total circulating protein appears to be much closer, as indicated by Fig. 3. With small changes in plasma volume there is no consistent variation in the total circulating protein. It is also apparent that in many cases the fall in total circulating protein with a lowered plasma volume was not as great as the rise in total circulating protein with a proportional elevation in plasma volume. Large changes in the plasma volume were generally associated with large changes in the total circulating protein. A fairly close relationship might be described for the combined groups with a correlation coefficient of 0.85 and an error of estimate from a linear regression line of 6.6 per cent. It would appear that the percentage change in total circulating protein will be approximately 0.6 ± 6.6 of the percentage variation in plasma volume.

DISCUSSION

From these observations the changes in the concentration of the serum proteins fail to reflect quantitatively the alteration in the plasma volume as measured by a dye dilution method, though an inverse directional change is usually present. It is apparent from inspection of Figs. 1 and 2 that with increases in plasma volume, the relative change of the serum protein concentration is considerably less than it is with decreases in plasma volume. In both instances, however, the change in the serum protein concentration is less than the change in plasma volume. This may be explained by the relative change in the total circulating protein which is increased with elevation of the plasma volume and decreased as the plasma volume becomes smaller. It would appear that serum protein may be actually added to the blood stream with dilution of the plasma and taken from the blood stream as the plasma becomes more concentrated. Thus it would be impossible for the concentration of serum protein to reflect accurately alterations in the plasma volume, since the total amount of protein present in the plasma is not constant under these conditions.

Similar observations have been made in man by others using different dye methods or the carbon monoxide method for the estimation of plasma volume. Bazett and co-workers,[8] in a study of the effects of environmental temperature, noted a maximal increase in plasma volume of 29.5 per cent as measured by a Congo red dye, with a change in the serum protein concentration of 3.4 per cent. With the carbon monoxide method, a fall in the plasma volume of –31 per cent was associated with a rise in the serum protein concentration of only 5.4 per cent. Maxfield and associates,[9] in a similar study using a modified carbon monoxide method, noted a maximal increase in plasma volume of 31.3 per cent with a decrease in serum protein concentration of only 5.2 per cent. Calvin, Decherd, and Herrmann,[10] using an indirect dye method for measuring plasma volume, noted a much greater decrease in the plasma volume than could be accounted for by the change in serum protein concentration.

The concept that the protein content of the blood stream is not static but may undergo considerable change, proposed by Madden and Whipple, has been given support by others who have found small changes in serum protein concentration following large additions to, or subtractions from, the total circulating protein by means of plasma infusions or phlebotomy.

It has been demonstrated that following phlebotomy there is a rapid return of the total circulating protein and that shifts of the serum protein concentration are considerably less than would be expected from the change in the plasma volume determined by dye methods[11-14] or by changes in the concentration of hemoglobin.[15] It has also been shown that with repeated large serum infusions the serum protein concentration does not increase as much as might be expected from the amount of protein added to the circulation and the relatively small change in the plasma volume.[16]

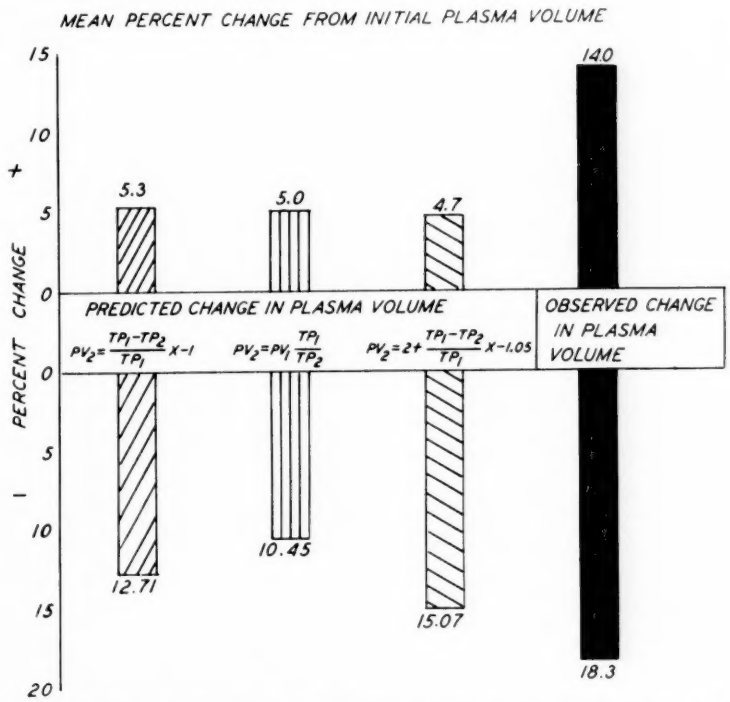

Fig. 4.—Prediction of the mean percentage change plasma volume (*1*) from the percentage change in serum protein concentration, (*2*) from the ratio of the initial plasma volume to alteration in serum protein concentration and (*3*) from a linear regression formula, compared to (*4*) the observed change in plasma volume.

The ability of the plasma protein to pass into or out of the circulation depends on the state of the protein reserves. When these have been depleted, less protein may be mobilized in the circulation and added serum may be more readily lost from the total circulating protein.[16, 17] The normal subjects in this study were, as far as could be determined, well nourished and presumably had adequate protein reserves. There was, however, considerable individual variation in the response of the serum proteins under similar conditions.

The increase in the total circulating protein in the subjects with an increase in the plasma volume is similar in many respects to the addition of circulating protein to the blood stream after a phlebotomy. In either situation there is a dilution of the plasma which may be the stimulus for the addition of more protein to the blood stream. Such an effect has been demonstrated by Shearburn[17] in dogs given a saline infusion which resulted in an increase in the total circulating protein as the plasma volume was increased. A similar analogy may be made between the decrease in the plasma volume resulting from dehydration and the injection of hypertonic serum. Although there is a rise in the serum protein concentration with dehydration, the total circulating protein decreases.

It would appear that it is not the change in the plasma volume per se that controls the quantity of circulating protein but rather the alteration in the concentration of the serum protein so that the osmotic equilibrium between the plasma and the extracellular fluid is not greatly disturbed by large changes in the plasma volume.

It was evident in these subjects that the concentration of serum protein paralleled the decreases in plasma volume associated with dehydration more closely than with increases in plasma volume. With the loss of extracellular fluid in dehydration there is also a decrease in the tissue pressure with its resultant effect on the capillary osmotic equilibrium. Thus a relatively greater concentration of serum protein would be required to maintain the depleted plasma volume. It is also possible that these normal subjects are less able to remove readily excess protein from the blood stream than are hypoproteinemic subjects. On the other hand, with retention of water and salt the increased volume of the extracellular fluid may play a role in maintaining the elevated plasma volume and the concentration of serum protein might be expected to fall considerably. However, it would appear that sufficient protein is added to the plasma so that the osmotic equilibrium between plasma and interstitial fluid is not greatly altered and the added protein also serves to maintain the elevated plasma volume.

Because of the change in the total circulating protein, prediction of the plasma volume based on variations in the concentration of serum protein will underestimate the plasma volume. In Fig. 4 are illustrated such predictions of the mean percentage change in the plasma volume based on the percentage change in serum protein concentration (Column 1), on the ratio of the initial plasma volume to the alteration in serum protein concentration (Column 2), and on the line of regression for these cases (Column 3) compared to the observed change for these cases (Column 3) and compared to the observed change in the plasma volume (Column 4).

Although approximation of the change in the plasma volume may be made from variation in the serum protein concentration, it should be remembered that such approximations tend to underestimate the changes, especially when the plasma volume is increased. The error of such predictions is sufficiently large, however, to suggest that the use of alteration in the serum protein concentration in evaluating changes in plasma volume in experimental observations is not justified, especially in instances where the change in plasma volume is relatively small.

SUMMARY

1. A comparison has been made between changes in the plasma volume, serum protein concentration, and total circulating protein in eighty-five studies on normal subjects.

2. The serum protein concentration is not altered in proportion to the change in the plasma volume due to the fact that protein is added to the plasma with increases in plasma volume or taken from the plasma with decreases in the plasma volume.

3. Prediction of the plasma volume from alteration in serum protein concentration tends to underestimate the degree of change in plasma volume and is associated with considerable error.

We wish to express appreciation to Drs. F. N. Wilson, H. C. Carver, and P. S. Dwyer for their patient assistance in the statistical arrangement of the data.

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PRECIPITIN TEST FOR TUBERCULIN ANTIBODIES*

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THE use of tuberculin in the diagnosis of tuberculosis has not been too satisfactory since it is not, strictly speaking, a quantitative test, and the amount of antibody which develops in the patient cannot be followed easily. The complement fixation test, too, has not been satisfactory.

In 1940, Cannon and Marshall[1] showed that the sensitivity of the precipitin test can be enhanced by the use of antigen-coated particles, and Weir[2] applied this technique to the demonstration of antibodies against tuberculin. However, the difficulties inherent in the production of uniform collodion particles make this test technically difficult. In 1941, Roberts and Jones[3] demonstrated that

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killed bacteria cells could be coated with antigen and that such antigen-coated cells made possible the detection of extremely small amounts of antibody. Their studies have been limited to virus diseases and so far as can be determined, the principles they described have not been applied to other antigens. It was decided to attempt to coat bacteria with tuberculin for the purpose of developing a quantitative precipitin test against tuberculin.

PROCEDURE

Preparation of Bacterial Suspension.—*Serratia marcescens* were used because of their small uniform size. One cubic centimeter of a twenty-four hour broth culture of the bacteria was seeded onto plain agar in a Kolle flask and incubated for twenty-four hours. It was then washed off with physiologic saline adjusted to a pH of 6. The bacterial suspension was centrifuged and washed twice with the physiologic saline. After the final washing the bacteria were suspended in 20 c.c. of neutral distilled water and killed with flowing steam. This is the stock cell suspension and will keep indefinitely if stored in a refrigerator at 8° C.

Preparation of Old Tuberculin Solution.—Standard commercial old tuberculin solution (Mulford) was used. The tuberculin was diluted to 0.5 per cent by placing 0.5 c.c. of the old tuberculin in a 100 c.c. sterile volumetric flask and diluting to the mark with sterile physiologic saline. This solution is used as a stock solution and will keep for months if stored in the refrigerator.

Preparation of the Antigen.—The nitrogen content of the stock cell suspension and of the 0.5 per cent old tuberculin solution was determined by acid and superoxol digestion and nesslerization. The cell suspension was diluted so that the nitrogen content was the same as that of the dilute old tuberculin. Equal portions of each, that is, dilute cell suspension and 0.5 per cent old tuberculin, were mixed and incubated at 37.5° C. for twelve hours. The old tuberculin was adsorbed on the cells in this interval. The mixture was then centrifuged and the coated cells thrown down. These were washed twice in saline to remove any excess or free old tuberculin. The coated cells were finally suspended in physiologic saline (pH 6) to a density equivalent to a reading of 70 on the Klett-Summerson photoelectric colorimeter, using a green filter (540 mu). This suspension was used as the antigen for the test.

Test.—The test proper was done as follows:

Blood from the subject was obtained in the usual manner, observing aseptic precautions. The blood was placed in a sterile tube and, after clotting, the serum was carefully removed. It may be stored in the cold indefinitely if free of contamination.

Serial dilutions were made of the serum ranging from 1:8 to 1:2,048 or more. Then 0.5 c.c. of antigen was added to 1 c.c. of each of the serum dilutions. The tubes were shaken vigorously for five minutes (Kahn shaker may be used) and incubated at 37.5° C. for two hours. The test was read at this time, then placed in a refrigerator at 8° C. for twelve hours, and reread after being allowed to warm up to room temperature.

Positive tests were manifested by a fine granular precipitate best read against a blue light in a darkened room.

Suitable controls were carried out using untreated cells and diluted serum as well as antigen and saline controls. During the early stages of the study a cell suspension control was carried out in all dilutions, but in later work

only the 1:8 serum dilution was used. These two control tubes, namely, coated cells and saline and uncoated cells and serum, should always be included for each test.

It is imperative that the following precautions be observed:

1. Antigen, serum, and saline must be sterile.

2. Antigen, that is, coated cells, must be prepared daily or be kept frozen in a deep-freeze refrigerator at –20° C.

3. The test must not be left in the refrigerator for more than twenty-four hours or false positive tests will result.

4. The precipitate is extremely fine and requires great care and considerable skill for accurate reading.

RESULTS

Studies on Guinea Pigs.—Twelve guinea pigs, after having been bled from the heart for control test, were inoculated into the liver with concentrated material known to contain tubercle bacilli. Two other guinea pigs were used for controls throughout the experiment. All animals were bled at weekly intervals after the preliminary or control bleeding.

The results are given in Table I. The preliminary bleeding and guinea pig controls were negative throughout.

TABLE I

DEVELOPMENT OF AGGLUTININ AGAINST TUBERCULIN IN EXPERIMENTALLY INFECTED GUINEA PIGS

GUINEA PIG	CONTROL	FIRST WEEK	SECOND WEEK	THIRD WEEK
1	Neg.	Neg.	1:4	1:4
2	Neg.	Neg.	Neg.	Neg.
3	Neg.	Neg.	1:2	1:2
4	Neg.	1:4	1:8	1:16
5	Neg.	1:2	1:4	1:16
6	Neg.	Died	---	----
7	Neg.	1:128	1:8	1:32
8	Neg.	1:16	1:32	1:32
9	Neg.	1:8	1:16	1:32
10	Neg.	1:32	1:32	1:128
11	Neg.	1:8	1:64	Died
12	Neg.	1:16	1:64	1:128
Control	Neg.	Neg.	Neg.	Neg.
Control	Neg.	Neg.	Neg.	Neg.

One animal of the twelve injected failed to develop antibodies and one animal died during the first week of the experiment. The other ten developed antibodies but the titers varied from 1:2 to 1:128. It is possible that the low titers were the result of the overwhelming infection. This point is now under study.

Studies on Tuberculin-Negative and Tuberculin-Positive Patients.—A group of individuals were skin tested with old tuberculin and a sample of blood drawn from each for agglutination test. The blood was drawn before the skin test so that there could be no question of the antibody titer being disturbed by the old tuberculin.

The sera of eight tuberculin-positive and eight tuberculin-negative patients were then subjected to the agglutination test with results given in Table II.

It will be noted that only one tuberculin-negative individual had antibodies in the serum while all of the tuberculin-positive individuals had positive tests, although the titer was not high in any of these, ranging from 1:16 to 1:64.

TABLE II

AGGLUTININ TITER AGAINST TUBERCULIN IN TUBERCULIN-POSITIVE AND TUBERCULIN-NEGATIVE INDIVIDUALS

PATIENT	TUBERCULIN NEG.	PATIENT	TUBERCULIN POS.
1	Neg.	1	1:16
2	Neg.	2	1:16
3	1:128	3	1:32
4	Neg.	4	1:32
5	Neg.	5	1:32
6	Neg.	6	1:64
7	Neg.	7	1:64
8	Neg.	8	1:64

Studies on Patients With Active Tuberculosis.—Thirteen sera were furnished us by the staff of the Mount St. Rose Sanatorium. No clinical histories were submitted. The results are given in Table III. In every case the serum contained antibodies, and in all but four of the thirteen sera the antibody titer was 1:256 or higher. Since this sera was from an unselected group of patients, the possibility that some of them were well on the way to recovery must be considered, and with this in mind the next phase of the study was undertaken.

TABLE III

AGGLUTININ TITER AGAINST TUBERCULIN IN ACTIVE AND ARRESTED CASES OF TUBERCULOSIS

ACTIVE TUBERCULOSIS		ARRESTED TUBERCULOSIS	
PATIENT	TITER	PATIENT	TITER
1	1:64	1	1:32
2	1:64	2	1:64
3	1:128	3	1:64
4	1:128	4	1:64
5	1:256	5	1:64
6	1:256	6	1:64
7	1:256	7	1:128
8	1:256	8	1:128
9	1:512	9	1:128
10	1:512	10	1:128
11	1:512	11	1:256
12	1:1024	12	1:256
13	1:1024		

Studies on Patients With Arrested Tuberculosis.—The twelve sera studied were from patients in the outpatient department of the Firmin Desloge Hospital who had been under observation for from one to five years. The results are given in Table III. All the sera contained antibodies, but, as was to be expected, the titer in all instances was relatively low. Only two of the twelve sera had an antibody titer above 1:256. Our experience at present is too limited to determine whether or not an elevated titer such as is seen in these two cases foretells of impending activity or not. It seems quite possible, however, that routine examinations of this kind might be a satisfactory method for detecting early recurrence of activity in patients with arrested tuberculosis.

CONCLUSIONS

A relatively simple method for the quantitative detection of agglutinins in tuberculosis which is apparently sensitive and specific has been presented. The results obtained suggest that the antibody titer is significantly different in active and inactive tuberculosis and that individuals who do not react to old tuberculin by skin test will not have antibodies in the serum.

This study is of a preliminary nature but seems to be of sufficient constancy and interest to justify its presentation.

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THE SEROLOGIC DIAGNOSIS OF ENDEMIC TYPHUS

II. A Comparison of Water-Bath and Icebox Fixation in the Complement Fixation Test

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Introduction.—Complement fixation has been utilized in the study of a number of rickettsial diseases; for example, Rocky Mountain spotted fever,[1] Tobia fever of Colombia (Rocky Mountain spotted fever), "Q" fever of North America and Australia,[2] and endemic and epidemic typhus.[3-6] The reaction has been studied especially by Bengtson[3] in an attempt to determine its usefulness in the diagnosis of endemic typhus in man and by Brigham and Bengtson[7] in a comparison of various tests as means of diagnosis of the disease in experimentally and naturally infected rats. From the work of Bengtson it was concluded that the test was useful "in detecting recent and also past infection with endemic typhus virus" and that "the sensitivity of the test is indicated by the results with sera from cases in which infection occurred as long as nine years ago and as recently as seven days." Likewise, the studies of Brigham and Bengtson indicated that "the complement fixation reaction as applied to rat control programs should be a valuable aid in preventing the spread of endemic typhus to human beings."

The value of any serologic procedure as an aid in diagnosis depends on the two factors of specificity and sensitivity. Obviously, it matters little how sensitive a test is if it lacks specificity, and at the same time, its usefulness is limited if its sensitivity is so low that it fails to give reactions except with relatively high titer serum.

The Test.—The technique of the complement fixation test in rickettsial diseases has been described by Bengtson.[8] The reagents include guinea pig complement, anti-sheep cell rabbit hemolysin, and sheep red cells, antigen, and test serum. The antigen is a suspension of rickettsiae grown in the yolk sac of fertile hen eggs, following the method of Cox,[9] but this specially prepared suspension is not essential, as Damon and Johnson[10] have shown that commercial typhus vaccines are satisfactory substitutes for it.

Sera are inactivated for one-half hour at 56° C. and dilutions made in two-fold steps ranging from 1:4 to 1:512 or higher, if necessary, to reach the end point. Two-tenths cubic centimeter of serum with 0.2 c.c. of the proper dilution

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of antigen (containing 4 units) and 0.2 c.c. of complement (2 units) are combined and incubated for one hour at 37° C. in a water bath. Subsequently, 0.4 c.c. of a 2 per cent sensitized sheep cell suspension (containing 2 units of hemolysin in 0.2 c.c.) is added. The tests are then reincubated for one hour in the 37° C. water bath, placed in the cold room overnight, and read the following morning. The degrees of fixation are recorded as 4+, 3+, 2+, 1+, and trace, and the titer is considered to be the highest dilution showing 3+ or 4+ fixation. Serum, antigen, and hemolytic system controls are always included.

With the technique described, the results previously reported[10] were obtained.

Purpose of This Study.—The purpose of the present study was to extend the observations previously reported[10] by making a comparison of the results obtained when fixation was carried out at 37° C. in a water bath for one hour and at 6 to 8° C. overnight in the cold room. Our conclusions are based on the end points reached in determining antigenic titers of specially prepared rickettsial suspensions, commercial vaccines, diagnostic human serum from cases of suspected typhus, and rat sera collected in connection with the typhus control program.

Results.—The end points reached in the titration of specially prepared rickettsial suspensions and vaccines, when fixation was carried out in the water bath and the cold room, are shown in Table I.

From Table I it is evident that it made no difference whether the product was a specially prepared rickettsial suspension or a commercial vaccine; the antigenic titer was always higher by icebox fixation. The significance of this

TABLE I

ANTIGENIC TITERS OBTAINED WITH SPECIALLY PREPARED RICKETTSIAL ANTIGENS AND VACCINES WHEN FIXATION WAS ACCOMPLISHED BY ONE-HOUR INCUBATION IN THE WATER BATH AT 37° C. AND OVERNIGHT IN THE COLD ROOM

SOURCE OF ANTIGEN	STRAIN OF RICKETTSIA	ANTIGEN DILUTION												
		37° FIXATION						ICEBOX FIXATION						
		1:4	1:8	1:16	1:32	1:64	1:128	1:4	1:8	1:16	1:32	1:64	1:128	1:256
National Institute of Health	Endemic, Strain 82	4	4	4	2	–	–	4	4	4	4	4	2	–
	Endemic, Strain 84	4	4	4	4	–	–	4	4	4	4	4	2	–
Parke, Davis & Co. Vaccine														
792	Epidemic	4	4	1	–	–	–	4	4	4	3	–	–	–
793	Epidemic	4	4	3	–	–	–	4	4	4	4	2+	–	–
794	Epidemic	4	3	–	–	–	–	4	4	4	2	–	–	–
795	Epidemic	4	4	2	–	–	–	4	4	4	4–	–	–	–
Lederle Laboratories, Inc. Vaccine														
719	Epidemic	4	4–	3	–	–	–	4	4	4	4–	2–	–	–
721	Epidemic	4	4	4	1	–	–	4	4	4	4	4–	1	–
725	Epidemic	4	4–	3–	–	–	–	4	4	4	4–	–	–	–
726	Epidemic	4	4–	–	–	–	–	4	4	4	1	–	–	–
739	Epidemic	4	4–	2	–	–	–	4	4	4	4–	–	–	–
43	Epidemic	4	4–	3	–	–	–	4	4	4	4	2–	–	–
45	Epidemic	4	4–	3	–	–	–	4	4	4	4	2–	–	–
50	Epidemic	4	4–	1	–	–	–	4	4	4	4–	–	–	–

TABLE II

TITERS OBTAINED WITH PATIENTS' SERA WHEN EXAMINED BY THE WEIL-FELIX, WATER-BATH AND ICEBOX COMPLEMENT FIXATION TESTS

DATE	PATIENT	WEIL-FELIX END TITER	SERUM DILUTION															
			37° C. FIXATION								ICEBOX FIXATION							
			1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
12/22/44	?	4+ 1,280	4	4	4	4	4	4	tr	–	4	4	4	4	4	4	4	2
12/28/44	C. A.	4+ 160	4	4	4	4	4	4–	tr	–	4	4	4	4	4	4–	2	–
1/ 3/45	S. A. F.	4+ 320	4	4–	2+	–	–	–	–	–	4	4	4	4–	1	–	–	–
1/ 1/45	E. L. K.	?	4	4	4	4	4	1	–	–	4	4	4	4	4	4	2	–
1/ 6/45	V. E.	4+ 160	4	4–	4–	1	–	–	–	–	4	4	4	4	4	3	–	–
1/17/45	W. E. K.	4+ 160	4	4	4	4	4	4–	–	–	4	4	4	4	4	4	4–	–
1/20/45	F 5021	4+ 2,560	4	4	4	4	4	4	3	–	4	4	4	4	4	4	4	3
1/23/45	F. G.	4+ 640	4	4	4	4	4	3+	–	–	4	4	4	4	4	4	4–	–
1/ 2/45	D. W.	4+ 40	–	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–
1/13/45	D. W.	4+ 2,560	4	4	4	4	4	4	4–	–	4	4	4	4	4	4	4	Trace
1/11/45	W. W. Y.	4+ 80	–	–	–	–	–	–	–	–	2+	–	–	–	–	–	–	–
1/15/45	W. W. Y.	4+ 1,280	4	4	4	3	–	–	–	–	4	4	4	4	4–	–	–	–

TABLE III

RESULTS OBTAINED WITH RAT SERA IN COMPLEMENT FIXATION TESTS CARRIED OUT AT WATER-BATH AND ICEBOX TEMPERATURE

LAB. NO.	SERUM DILUTION															
	37° C. FIXATION								ICEBOX FIXATION							
	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
76	–	–	–	–	–	–	–	–	2	1	–	–	–	–	–	–
2	4	4	4	4	–	–	–	–	4	4	4	4	4	4	3	2
3	4	4	4	4	4–	–	–	–	4	4	4	4	4	4	4	2
8	4	4	–	–	–	–	–	–	4	4	4	4–	–	–	–	–
15	1	–	–	–	–	–	–	–	3	2	1	–	–	–	–	–
19	4	4	4–	–	–	–	–	–	4	4	4	4	4	2	–	–
21	4	4	4	4	4	–	–	–	4	4	4	4	4	4	4	2
22	4	1	–	–	–	–	–	–	4	4	4	2	–	–	–	–
23	–	–	–	–	–	–	–	–	3	2	–	–	–	–	–	–
25	1	–	–	–	–	–	–	–	4	2	–	–	–	–	–	–
32	4	4	–	–	–	–	–	–	4	4	4	3	–	–	–	–
39	–	–	–	–	–	–	–	–	4	3	–	–	–	–	–	–

observation lies in the fact that by using this technique a greater dilution of the antigen may be made and for a given number of tests a smaller amount of the undiluted antigen will be required.

In Table II are shown the results obtained when diagnostic human sera were titered by both procedures. As a matter of interest, the Weil-Felix end titers are included, together with the complement fixation results in two instances where second specimens were requested. In every instance it is obvious that the icebox test is more sensitive, as indicated by the degree of fixation obtained or the dilution of patients' sera in which a reaction was observed. In the two cases cited in which second specimens were submitted by the doctor, this difference in sensitivity is especially noticeable. Both of these sera were completely negative on first examination when tested by the water-bath procedure, but both gave a suspicious reaction in the icebox test. The validity of these suspicious tests is shown by the results when the second specimens were examined, as findings in both patients were positive in high dilutions of the serum. Such results are, of course, highly significant in a diagnostic laboratory (Table II).

Finally, there is to be considered the matter of the examination of blood from wild rats, as this is especially important in epidemiologic and control studies. The use of the Weil-Felix reaction in this connection has been reviewed by Brigham and Bengtson,[7] who concluded from their own work that the test was, at most, infrequently positive in the areas studied. On the other hand, they remarked that the complement fixation test "as applied to rat control programs should be a valuable aid in preventing the spread of endemic typhus to human beings." With this in mind, then, it is highly important to use the most sensitive technique available, and the results we have obtained with rat sera using water-bath and icebox fixation are shown in Table III.

As was the case with human sera, the results from rat sera indicate in all instances a stronger reaction in the tests when fixation was accomplished at icebox temperature. By this technique it also appears that in certain specimens a degree of reaction may be anticipated, although the same sera are completely negative when tested at water-bath temperature.

CONCLUSIONS

With the technique now recommended, comparative complement fixation tests, carried out at water-bath and icebox temperatures, yield results definitely in favor of the latter. In some instances the titers of the sera are considerably higher, in other instances some degree of reaction is obtained though parallel tests at water-bath temperature are completely negative, and in all instances the titers have been at least equal to those obtained at 37° C. This greater sensitivity of the icebox technique is especially important in a diagnostic laboratory in detecting sera having minimal concentrations of antibodies as shown in the cases cited where second specimens showed markedly rising titers.

In our experience no difficulty with anticomplementary reactions has been encountered except in sera that were old or possibly contaminated, and the controls have always been satisfactory. It is not our intention to imply, however, that the technique cannot be improved, and studies now in progress are directed toward that end. It is our belief, however, that in a diagnostic laboratory complement fixation for the detection of endemic typhus infection is best carried out at icebox temperature.

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COMPARISON OF IN VITRO AND IN VIVO PENICILLIN RESISTANCE OF A STRAIN OF HEMOLYTIC STAPHYLOCOCCUS AUREUS

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TESTS in vitro[1] to determine the sensitivity to penicillin of organisms cultured from patients with infections have been useful in the selection of cases suitable for treatment with penicillin and in the regulation of the course of treatment. The concentration of penicillin in the blood of patients injected intramuscularly every three hours with 25,000 units of this substance seldom rises above 0.1 units per cubic centimeter. Immediately after the administration, intravenously, of 10,000 units of penicillin, the level reaches little more than 1.0 unit per cubic centimeter. Tests in vitro with concentrations of penicillin in these ranges usually give evidence that the organisms being tested are either sensitive or resistant to the levels of penicillin in the blood of patients being treated.

Recently a strain of hemolytic *Staphylococcus aureus* which was resistant in vitro to concentrations of penicillin below 10 units per cubic centimeter was isolated from a patient with an abscess resulting from osteomyelitis of bones of the orbit. Penicillin was administered intramuscularly and the abscess was drained by surgical incision. The patient recovered promptly although cultures remained positive for the strain of hemolytic *Staphylococcus aureus* until the drainage from the wound ceased. The efficacy of penicillin in aiding the recovery of this patient, and the reliability of the sensitivity test in vitro were questioned. Further confirmation of resistance to penicillin of this strain of

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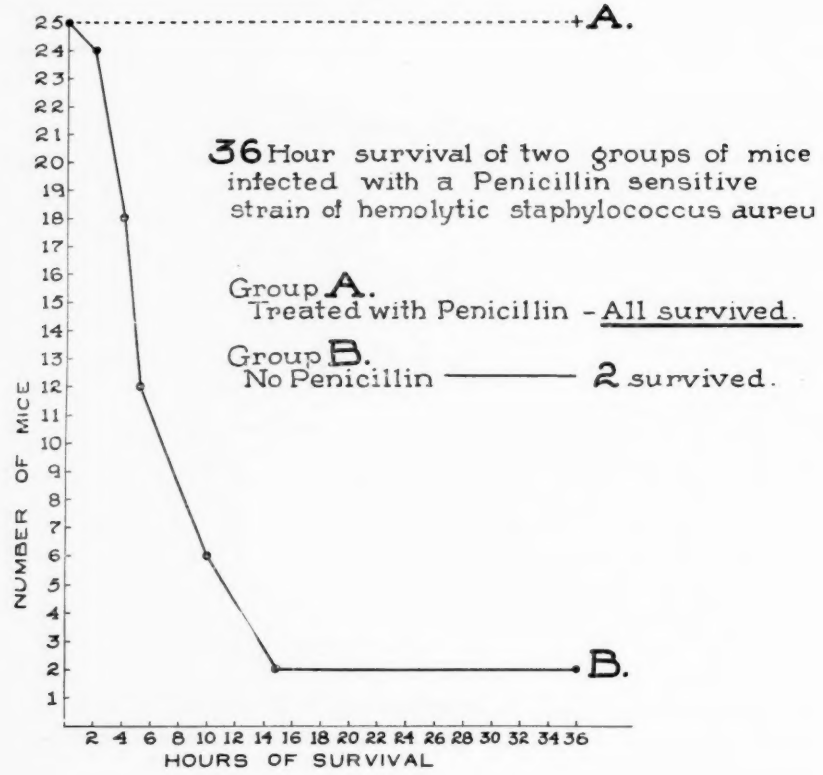
A.
36 Hour survival of two groups of mice infected with a Penicillin sensitive strain of hemolytic staphylococcus aureu
Group A.
Treated with Penicillin - All survived.
Group B.
No Penicillin ——— 2 survived.
B.
NUMBER OF MICE
HOURS OF SURVIVAL

Fig. 1.

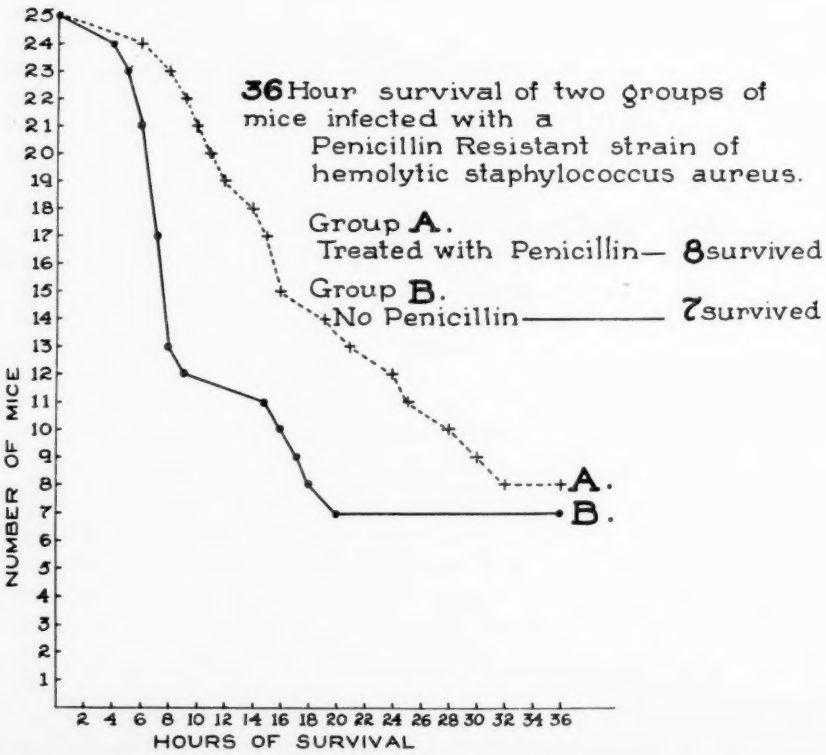
36 Hour survival of two groups of mice infected with a Penicillin Resistant strain of hemolytic staphylococcus aureus.
Group A.
Treated with Penicillin— 8 survived
Group B.
No Penicillin——— 7 survived
A.
B.
NUMBER OF MICE
HOURS OF SURVIVAL

Fig. 2.

hemolytic *Staphylococcus aureus* was sought by comparing its resistance to penicillin in vitro with experiments in vivo in mice.

Mice excrete penicillin more rapidly than human beings do, and it was found necessary to inject 200 units of penicillin per gram of body weight every one and one-half to two hours in order to obtain a level of about 1 unit of penicillin per cubic centimeter of blood. The mice varied from 10 to 30 grams in weight, the average being 15 grams. Each animal received 3,000 units of penicillin dissolved in 1 c.c. of physiologic saline intraperitoneally every one and one-half hours for the first four and one-half hours and less frequently thereafter as the infected mice developed edema. The remaining injections were given at intervals of from three to six hours, averaging about four hours. Half an hour after the third injection of 3,000 units the level of penicillin was 4.48 units per cubic centimeter of blood; three and one-half hours after the fifth injection it was zero. Rammelkamp's method[2] was used to measure the levels of penicillin in the blood.

For this experiment the cultures of hemolytic *Staphylococcus aureus* were grown for twenty-four hours on agar slants, washed off, and measured in Hopkins' tubes. Since the pathogenicity of staphylococci varies considerably in different strains, it was necessary to determine the dose that would be lethal to mice in about twenty-four hours. Ten billion *Staphylococcus aureus* organisms of this strain inoculated intraperitoneally were found to be the minimum lethal dose that would kill most of the mice in approximately twenty-four hours.

Each of fifty mice was inoculated intraperitoneally with 10 billion staphylococci of the strain resistant to penicillin; each of fifty others was inoculated with a similar dose of a strain of hemolytic *Staphylococcus aureus* sensitive to penicillin. Twenty-five mice in each group were injected with penicillin. The results are summarized in Figs. 1 and 2.

SUMMARY

In experiments using mice, a strain of hemolytic *Staphylococcus aureus* that was resistant to penicillin in vitro proved also to be resistant to comparable concentrations of penicillin in vivo.

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FURTHER STUDIES ON CHOLINE DEFICIENCY IN DOGS

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STUDIES on choline deficiency in young puppies have been reported from this laboratory.[1, 2] The principal lesion observed in this deficiency was an extensive fatty metamorphosis of the liver and was accompanied by marked impairment of liver function as measured by bromsulfalein elimination, plasma phosphatase, plasma cholesterol and cholesterol ester levels, and prothrombin time. These liver function tests are not specific for this particular condition but reflect parenchymal liver disease of various etiology. If more specific tests for choline deficiency were available, it would be of interest to apply them to patients with liver disease. Since an excess of methionine protects the pup from this deficiency, the primary defect is thought to be one of "labile methyl" rather than of choline per se.[1] It would appear that a method of testing the "methyl reserves" of the deficient and control animals might serve as a more specific index of the state of nutrition with respect to choline. The role of labile methyl from methionine or choline in the synthesis of creatine and creatinine in the rat, rabbit, and man has been summarized by du Vigneaud.[3] He has suggested that other nitrogen or sulfur-linked substances containing methyl groups present in tissues are dependent on dietary choline or methionine for their formation. Among these we have selected the N-methylated derivatives of nicotinic acid for study along with creatinine and creatine. In this paper we wish to present some observations on the excretion of these substances by choline-deficient and control puppies together with observations on improvement in liver function in choline deficiency following choline therapy.

EXPERIMENTAL*

Two litters of three puppies each were used in these experiments. All were given Ration 5[1] but containing 3 per cent cod-liver oil instead of 2 per cent and 1 per cent less sucrose. In addition, 20 mg. per cent of nicotinic acid was added to the ration of Dogs 38, 40, 41, and 42 in order to insure greater excretion of methylated derivatives of nicotinic acid. One pup in each of the two litters (Dogs 39 and 42) was given a supplement of 200 mg. of choline chloride per 100 Gm. of ration; the other two in each litter received only the basal ration. The dogs were all weighed and given water-soluble vitamin supplements orally each day and injections of Pitman-Moore antidistemper serum weekly as previously described.[1] Food consumption records were kept daily. The growth curves of these animals are given in Fig. 1. Dogs 38, 40, and 41 were given choline supplements after a suitable period on the deficient ration, and the return to normal liver function was observed by carrying out liver function tests every three or four days. Dog 38 was sacrificed on the tenth day after beginning the choline supplement, and the total lipids of the liver of this animal were determined. Liver function tests were obtained by methods previously described.[1]

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The dogs were housed in metabolism cages during the periods of study of the excretion of creatine, creatinine, and N methyl nicotinic acid derivatives. The daily urinary excretion of these substances was determined from suitable aliquots of filtered urine. Creatinine was determined colorimetrically using the alkaline picrate color measured in the Coleman spectrophotometer at 500 mμ. Creatine was determined in the same fashion after conversion to creatinine by autoclaving with 2N sulfuric acid. Total urinary derivatives of N methyl nicotinic acid were determined by the method of Sarett.[4]* In this method, N methyl nicotinamide substances are first converted to trigonelline by alkaline hydrolysis. The "total" trigonelline is then determined and thus all methylated nicotinic acid derivatives known to be present in dog urine are accounted for.

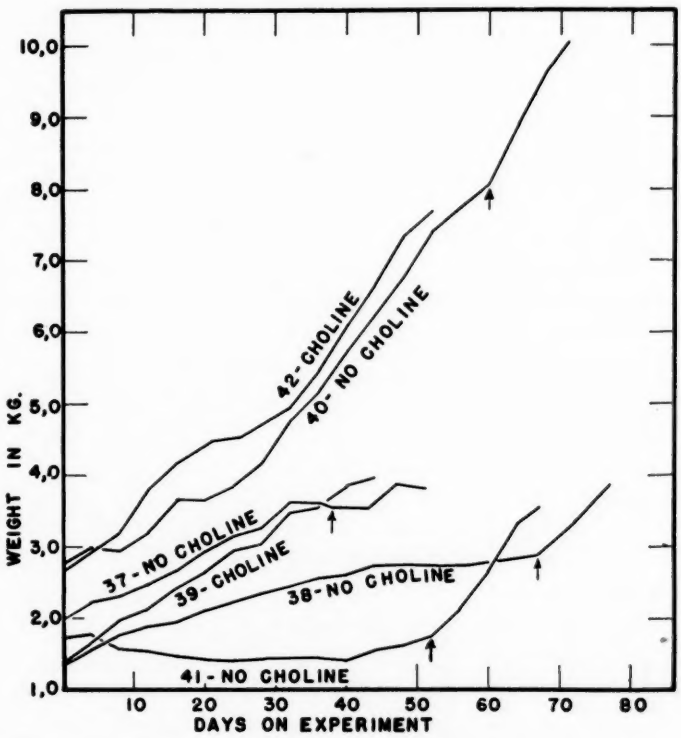

Fig. 1.—Growth curves of all the dogs used in these experiments. Arrows indicate beginning of period of choline supplement.

DISCUSSION

From Fig. 1 it can be seen that the two control dogs (Dogs 39 and 42) grew normally throughout the experimental period, although Dogs 37, 38, and 39 were a smaller, slower-growing breed than the others. The results of the liver function tests are given in Table I. It is seen that the control dogs gave normal results throughout the experimental period. Deficient Dogs 38 and 41 showed marked inhibition of growth and severe liver disease as judged by the liver function tests obtained on the sixty-eighth and fifty-third experimental days, respectively. Beginning on the sixty-eighth experimental day, 200 mg. per cent of choline chloride was added to the ration of Dog 38 and a total of 6.65 Gm. was given orally by gelatine capsule during the following ten days. This supplement produced striking improvement in liver function and all tests gave normal results by the tenth day. The dog was then sacrificed and the liver was found

*We are indebted to Dr. W. A. Perlzweig for generously furnishing us with trigonelline sulfate for use in this method.

TABLE I

SUMMARY OF LIVER FUNCTION TESTS OF ALL DOGS

Control Dog 39—200 mg. Per Cent Choline Chloride						
Experimental day		18		24		39
Plasma phosphatase		79		170		144
Bromsulfalein test						6
Control Dog 42—200 mg. Per Cent Choline Chloride						
Experimental day		18		39		53
Plasma phosphatase		232		321		192
Plasma phosphatase in 0.05 M NaCN				2		
Plasma phosphatase in 0.01 M NaCN						0
Bromsulfalein test		10				8
Plasma total cholesterol		160				240
Plasma cholesterol esters		99				198
Prothrombin time						8.9
Choline-Deficient Dog 37—Choline Started on Thirty-ninth Day						
Experimental day	18	24		39		44
Plasma phosphatase	853	1340		541		246
Bromsulfalein test				10		
Prothrombin time				12.4		
Choline-Deficient Dog 40—Choline Started on Sixty-first Day						
Experimental day	18	32	39	53	61	66
Plasma phosphatase	1040	593	641	763	586	223
Plasma phosphatase in 0.05 M NaCN		4	4			
Plasma phosphatase in 0.01 M NaCN				200	170	32
Bromsulfalein test	31	23		26	29	7
Plasma total cholesterol	116	169		166	158	231
Plasma cholesterol esters	69	91		86	99	165
Prothrombin time				10.9		
Choline-Deficient Dog 38—Choline Started on Sixty-eighth Day						
Experimental day	18	58	68	71	74	78
Plasma phosphatase	247	1435	1348	1088	651	357
Bromsulfalein test		26	38	20	12	7
Plasma total cholesterol		72	52	95	119	154
Plasma cholesterol esters		39	34	45	83	101
Prothrombin time			22.0	13.4	9.3	
Choline Deficient Dog 41—Choline Started on Fifty-third Day						
Experimental day		39	53	57	61	66
Plasma phosphatase		1164	1481	854	371	292
Plasma phosphatase in 0.05 M NaCN		0				
Plasma phosphatase in 0.01 M NaCN			425	342	90	50
Bromsulfalein test			27	9	7	7
Plasma total cholesterol			54	118	174	179
Plasma cholesterol esters			30	79	111	135

Plasma Phosphatase—Micrograms of inorganic phosphorus liberated per cubic centimeter of plasma in twenty-four hours. Normal range for control pups[1]: 80 to 350.
Bromsulfalein Test—Micrograms of dye per cubic centimeter of plasma at eight minutes. Normal range: 3 to 10.
Plasma Total Cholesterol—Milligrams per cent. Normal range: 100 to 250.
Plasma Cholesterol Esters—Milligrams per cent. Normal range: 70 to 200.
Prothrombin Time—Seconds. Normal range: 8 to 13.

to contain a normal amount of lipid material as determined by chloroform extraction (12.4 per cent of dry weight). This indicates a rapid removal of liver lipid since the liver would be expected to contain 40 to 50 per cent lipid (dry weight) before choline therapy, as judged from previous studies with this degree of impairment of liver function.

With Dog 41 the ration was supplemented with 200 mg. per cent of choline chloride beginning on the fifty-third experimental day and a total of 2.38 Gm. orally by gelatine capsule during the following five days. Rapid improvement in liver function resulted from this supplement and normal liver function was obtained by all methods of testing on the eighth day following the inception of choline therapy. Striking increases in food consumption and body weight were produced in both Dogs 38 and 41 by the choline supplements. In neither dog, however, did the addition of choline to the diet produce any significant

increases in the daily per kilogram output of creatinine or in the excretion of methylated nicotinic acid derivatives in terms of the per cent of the total nicotinic acid intake. Dog 38, however, did show a significant increase in the per kilogram excretion of creatine after five days of choline supplementation. This was not observed in Dog 41 and has not been observed in other dogs.

Dogs 37 and 40 showed slight early growth impairment on the deficient diet and subnormal liver function. Thus Dog 37 showed a moderately high plasma phosphatase value on the eighteenth day which rose to a very high value on the twenty-fourth day. However, growth ceased shortly after and perhaps accounted for the spontaneous improvement in liver function observed on the thirty-ninth day. At this time, the dog was given 0.5 Gm. of choline chloride orally and was placed on the control ration. The plasma phosphatase value was normal five days later.

Dog 40 showed a slight pause in growth between the fifteenth and twenty-fifth day and poor liver function as measured by plasma phosphatase and bromsulfalein elimination on the eighteenth day. Plasma cholesterol fractions at this time were not abnormally low, however. This animal showed a striking spontaneous improvement in liver function and gain in weight after this time although, as in the case of Dog 37, liver function did not return to normal. Beginning on the sixty-first day, the ration of this dog was supplemented with 200 mg. per cent of choline chloride. Again liver function tests were found to be normal five days later. No change in the creatinine excretion of Dog 40 was produced by the choline supplement, while the creatine excretion actually dropped slightly. The excretion of N methyl nicotinic acid derivatives was extremely variable in the two deficient periods studied and was somewhat elevated after the addition of choline to the ration. This spontaneous remission in Dog 37 and particularly in Dog 40 is remarkable and in both instances occurred in the largest dogs of the litter. We have previously observed "resistance" to choline deficiency (Dog 29, Experiment 5).[1] This dog, the largest of a litter of seven, showed good growth on an intake of only 5 mg. of choline per 100 Gm. of ration. It is possible that the choline requirement may be lowered after a certain stage in growth or development has been reached. The healing of kidney lesions in young rats surviving choline deficiency is a remarkable phenomenon and may be analogous to this conjuncture in the dog.

Judging from the changes in liver function produced by choline therapy in choline-deficient pups, it would appear that in severely deficient dogs (38 and 41), a week or ten days were required to produce normal liver function, whereas with less severe deficiency (Dogs 37 and 40) five days of therapy were sufficient. It must be emphasized, however, that rather large quantities of choline were given and that the liver disease was of a more acute than chronic nature. These factors would operate to give us a more rapid remission than would reasonably be expected under other circumstances. The index of liver function which gives the slowest return to the normal is the phosphatase level.

The effect of cyanide on the plasma phosphatase activity in these animals is of interest in connection with the findings of Drill and co-workers.[5] They observed that sodium cyanide in concentrations of 0.0001 to 0.1 M markedly inhibited the high serum phosphatase activity of dogs with hepatic damage produced by poisoning with carbon tetrachloride, by cholecystectomy, or by bile fistula. These concentrations of cyanide had only a slight inhibitory effect on normal dogs. They postulated that the increase in serum alkaline phosphatase in liver damage is due to an increase in a component phosphatase normally pres-

ent in only small amounts. We thought it would be of interest to see if this relationship would hold in the liver damage of choline deficiency. The addition of 0.05 M sodium cyanide to the substrate produced virtually complete inhibition of plasma phosphatase activity in Dogs 40, 41, and 42. The addition of 0.01 M sodium cyanide produced a marked inhibition in the plasma phosphatase activity of Dogs 40 and 41 and complete inhibition in Dog. 42. In Dog 41 the plasma phosphatase activity in the presence of cyanide is compared with the normal uninhibited activity throughout the course of remission following choline therapy. It can be seen that much of the high phosphatase activity of the deficient state is inhibited by cyanide but that the residual uninhibited portion is greater than that present in normal pups. As the plasma phosphatase activity decreases during the period of restoration of liver function, the cyanide insensitive portion decreases to a point far below the normal phosphatase activity. This was also observed in Dog 40. These results do not indicate a high specificity for plasma phosphatase components to cyanide inhibition since the activity may be completely inhibited by sufficient concentration of cyanide (0.05 M). If, indeed, there is a specificity at lower concentrations (0.01 M), it would appear that the plasma of the normal pup contains only a small fraction of cyanide-insensitive phosphatase activity which is proportionally increased along with the cyanide-sensitive components in severe choline deficiency. This does not imply a fundamental discrepancy between our results and those of Drill and co-workers[5] since they have used adult dogs and different techniques for the production of liver disease.

In Table II are given the results of the excretion of creatinine, creatine, and methylated derivatives of nicotinic acid. The excretion of creatinine was found to be constant in all the dogs and averaged from 18.7 to 22.6 mg. per kilogram of body weight daily in the various periods of study. We observed no daily variation in the creatinine output. The output of creatine varies greatly between different dogs, and we found considerable daily variation in the same dog. In general, the litter including Dogs 37 through 39 averaged a higher output of creatine per kilogram than did the litter including Dogs 40 through 42. The creatine output would appear, from our studies, to bear no consistent relation to the choline intake. Glycocyamine fed orally or injected subcutaneously did not appreciably increase either the creatine or creatinine output of Dogs 37 or 39. It seems clear from these studies that creatinine and probably creatine excretion are independent of dietary choline, as was found to be the case with muscle creatine content in our earlier studies. If creatine and creatinine formation do indeed depend on the labile methyl supply for their formation from glycocyamine,[3] there must be a high priority on the available methyl supply for their formation. This high priority forces a rather uneconomical drain on the methyl reserves when the creatine excretion is high, as in the case of one severely deficient dog (Dog 38), and this drain continues in the face of a progressively deteriorating liver function. This would indicate a lack of adaption on the part of the dog to a low methyl supply. However, it is also possible that synthesis of these substances in the dog may also take place from processes other than the methylation of glycocyamine by methionine or choline.

Our observations on the excretion of N methylated nicotinic acid derivatives are in general agreement with those of creatinine and creatine. The excretion of these substances expressed in terms of per cent of theoretical from the known nicotinic acid intake varies considerably from day to day and between dogs. Severely deficient dogs appear to excrete as much of these substances as they

TABLE II

SUMMARY OF EXCRETION STUDIES OF CREATINE, CREATININE, AND N METHYL NICOTINIC ACID DERIVATIVES

	LITTER 1				LITTER 2					
Dog	37	38		39	40			41		42
Supplement	None	None	Choline	Choline	None	None	Choline	None	Choline	Choline
Metabolism study (Days)	26	9	10	29	19	7	11	10	15	14
Days of experiment (Inclusive)	13-38	59-67	68-77	12-40	11-29	54-60	61-71	43-52	53-67	11-24
Average daily creatinine excretion (Mg./Kg.)	19.7	22.4	22.6	20.7	20.1	19.7	20.1	18.7	19.9	20.1
Average daily creatine excretion (Mg./Kg.)	41.1	28.8	47.3	38.1	26.3	18.0	13.1	12.3	14.1	13.1
Total nicotinic acid intake for period (Mg.)		178	346		673	418	898	132	515	545
Total nicotinic acid output as methylated derivatives for period (Mg.)		115	242		283	334	824	91	257	465
Per cent conversion of nicotinic acid to methylated derivatives		64.6	69.9		42.0	79.9	91.7	69.0	49.9	85.3

do when given choline. This is all the more surprising in view of the fact that the dog can also excrete nicotinic acid in the form of nicotinuric acid. This would be the expected form during a condition of methyl deficiency since no methyl is needed in this synthesis.

These observations question the possibility of testing the methyl reserves of dogs by observations of creatine or creatinine excretion or the excretion of methylated nicotinic acid derivatives following doses of nicotinic acid. Whether or not other substances which depend on labile methyl from choline or methionine for their formation would be useful in this connection remains to be determined.

SUMMARY

1. Addition of choline to the diet of the choline-deficient pup results in rapid increase in food consumption and weight, improvement in liver function, and withdrawal of lipid from the liver. Under the conditions of our experiments, normal liver function has been restored in from five to ten days.

2. Excretion of creatinine, creatine, and N methyl nicotinic acid derivatives does not appear to be influenced by choline deficiency in the young pup.

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AN ADRENAL CORTEX STIMULATING SUBSTANCE IN FEMALE HUMAN URINE

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THE presence of cortinlike substances in human urine has been demonstrated by Dorfman, Horwitt, and Fish.[1] These substances are capable of increasing the resistance of adrenalectomized rats to low environmental temperatures and of raising the mean liver glycogen concentration to five to ten times the normal amount.[2] It has been shown many times that there are substances in the urine and serum of pregnant women which act biologically in a manner similar to certain hormones of the anterior pituitary gland, but there are only a few reports that deal with hormones of this nature which might stimulate the adrenal cortex to elaborate the substances noted by the authors mentioned. Tepperman, Engle,

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*Now in the service of the U. S. Army.

and Long[3] refer to certain experiments in which de Boissezon[4] may have been successful in demonstrating such a material in the urine of pregnant women, although they state that this is not certain. More recently Golla and Reiss[5] have shown that pregnant mares' serum increases the adrenal weight of hypophysectomized rats and nine-day-old chicks; they postulate the presence in the pregnant mares' serum of a new corticotropic factor. No reference has been found which deals with a corticotropic factor in normal human urine.

The present experiments demonstrate the presence of a substance or substances in normal female human urine which, when injected into guinea pigs, produce an increase in mitotic activity in the adrenal cortex and in this respect acts similarly to certain substances elaborated by the anterior pituitary gland. The various types of urine used are shown in Table I. In most instances normal urine was obtained from women approximately five days before menstruation, although in a few instances it was obtained earlier in the sexual cycle. Pregnancy urine was collected from women three to three and one-half months pregnant. The urine was divided into two or three portions. The first portion was used as untreated fresh urine; to a second part, in some instances, thymol was added to act as a preservative; to a third portion formalin was added in sufficient quantity to bring the concentration of this substance in the urine to that shown in the various groups in Table I. Urine to which formalin had been added was then kept at a temperature of 39° C. and at pH 8 for seven days before injection into guinea pigs. The test animals consisted of seventy immature female guinea pigs ranging in weight between 170 and 210 grams which were injected intraperitoneally twice daily with 1.0, 1.25, or 2 c.c. of female human urine of the various types shown in Table I for periods of two and four days. Twenty noninjected immature female guinea pigs of a similar weight range served as controls. Mitotic counts in the adrenal cortex were carried out according to a method previously described[6] and were recorded as the average number of mitoses per section.

RESULTS

The results tabulated in Table I show three levels of mitotic activity in the adrenal cortex: (1) an average control level of 5.4 mitoses per section, (2) an average count in animals injected with untreated and with thymol- and formalin-treated pregnancy urine of 7.6 and 7.1 mitoses per section, respectively, and (3) an average of 12.6 mitoses per section in guinea pigs receiving untreated and formalin-treated normal female urine. The significance of the differences

TABLE I

THE EFFECT OF VARIOUS TYPES OF HUMAN FEMALE URINE ON MITOTIC ACTIVITY IN THE ADRENAL CORTEX OF IMMATURE FEMALE GUINEA PIGS

EXPERIMENTAL GROUP	NUMBER OF GUINEA PIGS	AVERAGE NUMBER OF MITOSES PER SECTION OF ADRENAL CORTEX		PROBABLE ERROR
1. Controls	20	5.4		±0.34
2. Normal female urine				
Untreated	10	12.3	12.6	±1.25
With 0.5 per cent formalin	5	13.1		±2.89
3. Pregnancy urine				
Untreated	16	8.2	7.6	±1.64
With thymol	12	6.8		±1.15
With 0.5 per cent formalin	12	6.2		±1.13
With 0.75 per cent formalin	9	7.9	7.1	±1.13
With 1.0 per cent formalin	6	7.8		±0.46

TABLE II

SIGNIFICANCE OF DIFFERENCE BETWEEN VARIOUS GROUPS*

EXPERIMENTAL GROUPS COMPARED	FACTOR OF STATISTICAL SIGNIFICANCE
1. Controls vs. normal female urine, untreated and with formalin	4.6
2. Controls vs. pregnancy urine untreated and with thymol	2.1
3. Controls vs. pregnancy urine with formalin	2.2
4. Normal female urine untreated and with formalin vs. pregnancy urine untreated and with thymol	10.2
5. Normal female urine untreated and with formalin vs. pregnancy urine with formalin	7.0
6. Pregnancy urine untreated and with thymol vs. pregnancy urine with formalin	1.7

*The factor of statistical significance was calculated from the formula $A_1-A_2/SE_{A1}-SE_{A2}$ in which A_1 and A_2 represent the averages of the groups compared and SE_{A1} and SE_{A2}, the standard errors of the respective groups. A factor of less than 2 is of no significance; a factor between 2 and 3 represents probability greater than chance, while one over 3 denotes an almost certain significant difference.

in results in these three groups has been tested in two ways. First calculations of the probable errors in all subgroups show that if we take into account these factors in comparing the results of the subgroups of one level with those of another level of mitotic activity, there is no overlapping of results. In the second place, the three major groups and various subgroups have been compared as shown in Table II. From the latter calculations it may be concluded that the differences obtained between animals injected with normal female urine and animals injected with pregnancy urine of various types are almost certainly significant, while the differences between the various groups receiving different types of pregnancy urine and controls are probably significant. On the other hand, treatment of both normal and pregnancy urine for twenty-four hours with various concentrations of formalin or, in the case of pregnancy urine, with thymol failed to alter significantly the results in these respective groups.

DISCUSSION

From these results it may be concluded that there is a substance present in the urine of sexually active nonpregnant women capable of producing a hyperplasia of the adrenal cortex of the guinea pig, and it is furthermore notable that such a substance is also present in the urine of pregnant women, although it may be there in smaller quantity or in association with some inhibitory substance, since the response is less marked than after the injection of normal female urine.

It is not possible to determine the origin of this substance from these experiments alone, although certain possibilities may be considered. In the first place, it may be that we are dealing with a metabolic derivative of the corticotropic factor of the anterior pituitary gland excreted in the urine which would then elicit a response in the adrenal cortex which is somewhat less than that observed with implants of fresh anterior pituitary substance. The response to normal female urine is only about half as great as that which we have observed with fresh human anterior pituitary gland in some as yet unpublished experiments. A similar relationship has been observed numerous times with respect to the estrogens elaborated by the ovary; in this case also certain urinary metabolites of estrogenic substances act in a manner qualitatively similar to, but qualitatively weaker than, the parent estrogens found in the ovaries. However, the factor of dilution in the urine must be considered before this conclusion can be made with certainty. To test this factor, in a few experiments a concentrated powder of pregnancy urine prepared by the method of Loeb and Hay-

ward[7] was injected into immature female guinea pigs. The results were not significantly different from those obtained with either fresh or formalin-treated pregnancy urine.

A second possibility which might be considered is that of a response of the adrenal cortex to ovarian hormones elaborated as a result of stimulating substances present in the urine. We have observed in some, as yet unpublished, experiments that administration of corpus luteum hormone produces an increase in mitotic activity in the adrenal cortex of the guinea pig, but this reaction is usually most marked in the zona glomerulosa as contrasted with that of anterior pituitary substance which induces mitotic activity almost exclusively in the outer half of the zona fasciculata. In our present experiments most of the mitotic figures were found in the outer half of the fasciculata, although in some experiments, specifically those with fresh pregnancy urine, there were in some cases as many as 15 per cent of the mitoses in the zona glomerulosa. This may be taken to indicate that the actual difference between normal and pregnancy urine is even greater than the results shown in Table I indicate, and that in the case of pregnancy urine there is in addition to the stimulus exerted by ordinary urine a stimulus of a different kind which affects the average number of mitoses per section in the glomerulosa. As further evidence against the assumption that luteal hormone is the agent present in the urine we might cite the observations of Loeb and Hayward,[7] that the luteinizing effect which pregnancy urine produces in the guinea pig ovary is inhibited by formalin treatment and replaced by a connective tissue ingrowth into the ovarian follicles. Thus, if luteal hormone were the major factor in inducing these proliferative effects, it should be expected that untreated pregnancy urine would call forth the greatest mitotic response in the adrenal cortex, since it produces the most marked luteinizing effect, and that treatment with formalin would diminish this response. It is shown in Table I that this is not the case, no essential difference being noticeable between normal and formalin-treated urine.

It is, therefore, most likely that we are dealing with a derivative of anterior pituitary substance or with some substance which acts in a similar manner. That this substance is different from the pituitary-like substance elaborated in the placenta is shown by the fact that normal female urine produces a greater response than pregnancy urine. It appears that in contrast with the conclusions of Golla and Reiss,[5] we are dealing with a substance which is not peculiar to the state of pregnancy but which occurs also in the urine of nonpregnant females and perhaps also in that of males, although the urine of males has not yet been studied from this point of view. This conclusion would still hold even if it were determined that in a later stage of pregnancy the urine is able to produce a mitotic response in the adrenal cortex greater than that observed after the injection of normal female urine. In a later stage the ovarian hormones might exert an influence sufficiently great to overcome such assumed antagonistic substances in the urine of pregnancy, or it is also conceivable that these latter substances present during the first trimester of pregnancy might gradually disappear as pregnancy progresses.

Finally, it would be emphasized that the demonstration of pituitary-like effects of some body fluid or of its excretion product in the urine on the adrenal cortex does not necessarily indicate that we are dealing with a new substance which has a specific corticotropic effect; it is more likely that this effect is due either to circulating pituitary hormone or to some metabolic derivative of it.

SUMMARY

The corticotropic effect of normal and pregnancy human female urine has been studied. It has been found that normal human female urine produces a greater increase in mitotic activity in the adrenal cortex of immature female guinea pigs than does pregnancy urine. Treatment of this urine with various concentrations of formalin failed to alter the results appreciably. The likelihood that this corticotropic effect of urine is due to a metabolite of anterior pituitary corticotropic hormone is discussed.

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THE DETERMINATION OF PROTHROMBIN TIME FOLLOWING THE ADMINISTRATION OF DICUMAROL, 3,3′-METHYLENEBIS (4-HYDROXYCOUMARIN), WITH SPECIAL REFERENCE TO THROMBOPLASTIN*†

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WHEN the Quick prothrombin time test was first used as a measure of bleeding tendency, particularly in hepatic disease, all the information that was actually necessary was whether the prothrombin time was normal or prolonged. The exact degree of prolongation or the exact amount of prothrombin deficiency was not particularly important. In recent years, the prothrombin time test has become the indispensable guide to the administration of dicumarol, 3,3′-methylenebis (4-hydroxycoumarin), and it is important to know not only the normal prothrombin time, but also the degree of prothrombin deficiency which has developed as a result of the dicumarol. In order that prothrombin times may give this information, it is necessary for the laboratory to standardize in detail the technique of the prothrombin time test as performed.

When prothrombin times are determined on prothrombin-deficient plasmas, it becomes apparent that the results obtained depend to a great extent on the activity of the thromboplastin employed, that in order to obtain consistent results from day to day it is necessary to employ thromboplastins of similar activity, and that to interpret adequately a particularly prothrombin time one must have certain information regarding the activity of the thromboplastin employed.

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Clinical studies[2, 3] have shown that after the administration of dicumarol, thrombosis almost certainly will not occur if the prothrombin time is greater than twenty-seven seconds (thromboplastin "A"* of this study, normal prothrombin time, seventeen to nineteen seconds); bleeding of any significant degree is very unlikely to occur among patients who have received dicumarol if the prothrombin time is less than sixty seconds (thromboplastin "A"). Arbitrarily a prothrombin time of thirty-five seconds (thromboplastin "A") was established by Allen, Barker, and Waugh[2] as a guide to the dosage of dicumarol as follows: On days when the prothrombin time was less than thirty-five seconds, a dose of the drug was given; on days when the prothrombin time was greater than thirty-five seconds, no drug was given. Obviously these rather critical points—twenty-seven seconds, thirty-five seconds, and sixty seconds—applied only to the test as performed and to the thromboplastic substance which was used (thromboplastin "A").

Meyer, Bingham, and Axelrod[24] expressed the opinion that prolongation of the prothrombin time with dicumarol to between twelve and one-half and nineteen seconds is reasonably safe. These workers used a thromboplastin of such potency that with an optimal solution of calcium chloride, the normal prothrombin time was 9.5 to 10.5 seconds.

Since the effect of thromboplastin on coagulation time (prothrombin time) is being considered, a brief review of the coagulation process may be in order.

Factors in Coagulation.—A number of factors are concerned in the coagulation of blood. These include prothrombin, thromboplastin, calcium, thrombin, and fibrinogen, as well as certain inhibitors, among which are plasma antithrombin, plasma antiprothrombin, and heparin. According to the most commonly accepted hypothesis, blood clotting may be regarded as a two-stage process:

1. Prothrombin + thromboplastin + calcium = thrombin
2. Thrombin + fibrinogen = fibrin

The exact mechanism of the first stage of clotting still remains a subject of controversy. Whether the conversion of prothrombin to thrombin results from an enzymic reaction whereby thromboplastin alone or in conjunction with calcium acts as a catalyst or whether there is an actual chemical combination of prothrombin with either thromboplastin or calcium or both is not known. It has been suggested that thromboplastin merely neutralizes antiprothrombin, thus allowing prothrombin to react with calcium, forming thrombin. Regardless of which, if any, of these hypotheses is correct, it is evident that thromboplastin greatly accelerates the speed of clotting.[28] Most workers have agreed that the presence of this factor in tissues and blood platelets is necessary to the conversion of prothrombin to thrombin under normal conditions. Aqueous solutions of tissue extracts,[28] suspensions of platelets,[8, 13] cephalin,[8, 16] snake venom,[14] and trypsin[15, 16] are known to accelerate clotting time.

The chemistry of thromboplastic substances and their effects is not fully known, though studies have been made. Eagle[14] has shown that the coagulative action of venom is probably due to a proteolytic enzyme. It is known that clotting activators from tissue cells may be thermolabile and soluble in water or thermostable and soluble in alcohol or ether. The phosphatide fraction from platelets has been found to contain a potent activator.[8] Both a protein and a phosphatide component of the thromboplastic protein from beef lung were re-

*See under Preparation of Thromboplastin.

quired for maximal thromboplastic action. The protein component was inactive after removal of the phosphatides.[10] Both "lecithin" and "cephalin" fractions of a complex phosphatide mixture extracted from the thromboplastic protein from lungs showed considerable clotting activity.[11] The "cephalin" fraction of brain phosphatides, formerly thought to be a definite compound, has been shown to be a mixture of phosphatides.[18] Fractional ultracentrifugation of saline extracts of beef lung has led to the isolation of a high molecular lipoprotein with high thromboplastic activity.[9] Chargaff,[7] in 1944, reported on the thromboplastic activities of numerous phosphatide fractions obtained from pig heart, beef heart, and beef brain. Phosphatidyl serine from beef brain was found to be inactive. Brain cephalin itself (that is, ethanolamine phosphoryl diglyceride) showed some activity. The conclusion was reached that "the thromboplastically active lipids cannot yet be identified with any of the known phosphatides."

Thromboplastic substances, aqueous tissue extracts,[12, 32, 37] and snake venom[19, 26, 27] in particular are used in laboratory tests for prothrombin. The thromboplastin employed has an effect on the rate of conversion of prothrombin to thrombin.

Methods for Prothrombin Determination.—The rate at which thrombin is formed is affected by the concentration of prothrombin. As the means of recognizing this factor is its capacity to form thrombin, any method for determination must be an assay based on formation of thrombin.[4]

The two-stage method of Warner, Brinkhous, and Smith[37] for prothrombin determination utilizes the biphasic nature of the clotting reaction. In the prothrombin conversion stage, prothrombin is converted completely to thrombin with an optimal amount of calcium and an excess of thromboplastin. In the clotting stage, the amount of thrombin formed is measured by the time required for the clotting of a standard solution of fibrinogen.

The one-stage method of Quick, Stanley-Brown, and Bancroft,[32] or some modification thereof, has been more widely adopted as a clinical test for prothrombin than the two-stage method. The one-stage method consists in determination of the clotting time of oxalated plasma at 37.5° C. after addition of an excess of thromboplastin and a fixed amount of calcium. Thus the time represents a summation of the conversion time and the clotting time. Quick stated that with the addition of a fixed quantity of calcium and an excess of thromboplastin, prothrombin is the only variable and the clotting time of the oxalated plasma can be considered a direct measure of the prothrombin concentration of the blood.

Dam and Glavind,[12] instead of determining prothrombin time with a fixed concentration of thromboplastin, determined the concentration of a tissue extract that would clot heparinized plasma in three minutes.

Factors Influencing the Prothrombin Determination.—Stewart and Pohle,[36] investigating the effect of calcium on the plasma prothrombin time, observed that the minimal coagulation time could be assured only by determining the optimal amount of calcium necessary for recalcification in each instance. Quick[30] agreed with them that clotting time could be shortened by reducing the strength of the calcium chloride, but he did not believe that calcium should be made a variable in the test.

Electrolyte concentration, pH, and temperature are known to influence the rate of conversion of prothrombin, and these factors should be kept constant in so far as is possible

Differences in "convertibility" of prothrombin have been observed by some workers. Whether this is due to species differences or to unrecognized factors which influence clotting is not known.[4] The presence of inhibitors also affects the conversion rate. "Assay methods for thromboplastin, inhibitors, and the 'convertibility factor' are still incompletely developed and a complete quantitative analysis of the clotting problem is not at present available."[34]

In stating the underlying principle of the prothrombin time test, the expression "excess thromboplastin" is generally used. This does not mean, however, that the presence of an excess makes up for any lack of potency of the particular thromboplastin employed. The prothrombin time obtained depends, in part, on the activity of the thromboplastic substance.

Factors Influencing the Activity of Thromboplastin.—The factor having the greatest influence on the activity of thromboplastin prepared from rabbit brain is whether or not the brain has been extracted with acetone. It will be noted that the thromboplastin "A" used routinely in this study has been made from dried rabbit brain without previous extraction with acetone. This follows the original method of Quick, Stanley-Brown, and Bancroft.[32] Many workers, however, prefer Quick's modified method[29] in which he extracted the brain with acetone previous to the drying process. With the latter method it was possible for Quick to prepare a more active thromboplastin (normal prothrombin time, twelve to thirteen seconds), whereas he had previously obtained a less active preparation (normal prothrombin time, twenty-two to twenty-five seconds*).

Unfortunately, thromboplastins prepared according to the same general method do not always possess similar activity. This is true regardless of the time which has elapsed between the preparation of the dried brain and preparation of the thromboplastin. It would not be profitable to enumerate every possible reason why individual preparations of thromboplastin may vary in activity. It should be realized, however, that the fineness of the powdered brain, the length of time and the temperature at which the saline extract is incubated, the amount of mixing prior to, during, and after incubation, and the period of centrifugation each may have an effect on the potency of the thromboplastin. When the brain is extracted with acetone, the length of time and the manner in which the extraction is carried out have an effect on the potency of the final thromboplastin. This effect may be due to differences in the amounts of either inactive or inhibitory substances removed by the acetone. Regardless of precautions taken, one may occasionally obtain a thromboplastin so different in activity from other preparations used that it is advisable to discard it.

The amount of dried brain extracted in the actual preparation of the thromboplastin may have considerable effect on the potency of the final strength, though the literature records considerable range in the initial concentration of the thromboplastins as prepared. Quick[28] has correctly stated that when the thromboplastic activity of increasing concentrations of a tissue extract was tested, the clotting time reached a minimum which could not be appreciably shortened by additional thromboplastin. One can go further, however, and say that as the concentration of thromboplastin is increased beyond the amount giving a minimal clotting time, the prothrombin time can be prolonged. This fact is not always evident when determinations are made on normal plasmas. But when thromboplastins of varying concentrations are tested with prothrombin-deficient plasmas, it will be noted that within certain limits of a selected prothrombin time, the time can be shortened or prolonged. This prin-

*Quick stated that thromboplastin prepared from rabbits obtained in a different locality would clot human plasma in sixteen to seventeen seconds.

ciple has a very practical application in determining prothrombin times on patients who are receiving dicumarol.

Preparation of Thromboplastin.—Thromboplastin "A" was prepared by a method similar to that previously described.[23] A rabbit was killed by injecting 15 c.c. of air into the lateral vein of the ear. The skull was quickly opened and the brain was removed, freed of meninges and blood, and then triturated with a spatula on a glass plate (7 by 10 inches [18 by 25 cm.]). It was then spread over the plate and allowed to dry in an incubator (37.5° C.) for approximately twenty-four hours. The dried brain was scraped off, care being taken to scrape in such a manner that it did not become too finely powdered. It was then stored at 5° C. in a tightly covered glass jar. It has not been found necessary to store the dried brain in ampules. As mentioned elsewhere, twenty-four dried brains were usually mixed together. The number of brains to be mixed together would naturally depend on the number of prothrombin time tests being performed by the individual laboratory.

The actual preparation of the thromboplastin was as follows: A selected amount of dried brain was added to a known volume of physiologic saline solution, the concentration depending on the activity of the material used.* The brain and saline solution were thoroughly mixed with the aid of a stirring rod, incubated at 56° C. for fifteen minutes, quickly cooled, again thoroughly mixed, and then centrifuged at low speed for not more than two minutes. The supernatant liquid is the "thromboplastin" and can be used as long as its activity is maintained. Obviously, the foregoing directions will be followed somewhat differently by different persons in so far as details are concerned, and thromboplastins of different activities may be obtained. This probably accounts in part for the variation in normal prothrombin time reported by various workers using the same prothrombin time test. However, the important thing is that each technician be consistent in the preparation of successive thromboplastins.

Selection of a "Standard" Thromboplastin "A".—The following procedure was adopted more than two years ago as a means of procuring thromboplastins of consistent activity. Thromboplastins were prepared from mixtures of the dried brains of twenty-four rabbits. Each mixture was kept at 5° C. and used over a period of approximately six weeks to two months. Each time a new mixture was tested, thromboplastins were prepared using 0.6, 0.8, and 1.0 Gm. of dried brain in 10 c.c. of saline solution. These preparations were tested with plasmas giving prothrombin times in the thirty-, forty-, and fifty-second range. Results obtained with two such mixtures are illustrated in Table I. The thromboplastin most nearly checking the one in use was selected (Table II). Concentrations ranging from 0.4 to 1.0 Gm. per 10 c.c. were sometimes used and it was usually possible to prepare a thromboplastin giving a somewhat shorter or longer prothrombin time, as was necessary to check a "standard" thromboplastin. A concentration of 0.6 or 0.8 Gm. per 10 c.c. was most often selected as being satisfactory. While normal plasmas were tested as well, it saved time and material to test plasmas with prolonged prothrombin times first.

The importance of checking each thromboplastin is shown in Table III. Unfortunately, thromboplastins prepared in like manner did not always possess similar activity. In this study each fresh preparation of thromboplastin "A" was checked with a previously "standardized" thromboplastin. Thrombo-

*See under Selection of a "Standard" Thromboplastin "A."

TABLE I

PROTHROMBIN TIME DETERMINATIONS TO SHOW COMPARISON OF THROMBOPLASTINS PREPARED WITH VARYING AMOUNTS OF DRIED BRAIN OF RABBIT

RABBIT BRAIN MIXTURE	THROMBOPLASTIN "A" (GRAMS IN 10 C.C. SALINE)	PROTHROMBIN TIME, (SECONDS)					
		NORMAL	PATIENTS				
			1	2	3	4	5
I	0.6	17	27	33	37	41	52
	0.8	17	29	36	41	47	59
	1.0	17	32	38	43	49	63
		NORMAL*	PATIENTS				
			6	7	8	9	10
II	0.6	18	18	26	38	47	54
	0.8	18	18	26	40	48	56
	1.0	18	18	28	45	53	65

*Different normal from that used with mixture I.

TABLE II

COMPARISON BETWEEN THROMBOPLASTINS PREPARED WITH VARYING AMOUNTS OF A NEW MIXTURE AND A PREVIOUSLY STANDARDIZED THROMBOPLASTIN

RABBIT BRAIN MIXTURE	THROMBOPLASTIN "A" (GRAMS IN 10 C.C. SALINE)	PROTHROMBIN TIME, (SECONDS)				
		NORMAL	PATIENTS			
			1	2	3	4
Old	0.7*	18	26	35	46	75
New	0.6	18	26	35	45	75
	0.8	18	28	38	54	87
	1.0	21	34	54	78	141

*Standardized.

TABLE III

VARIATION BETWEEN TWO THROMBOPLASTINS PREPARED SIMULTANEOUSLY AND TWO STANDARDIZED THROMBOPLASTINS

RABBIT BRAIN MIXTURE	THROMBOPLASTIN "A" (GRAMS IN 10 C.C. SALINE)	DATE OF PREPARATION	PROTHROMBIN TIME (SECONDS)					
			NORMAL	PATIENTS				
				1	2	3	4	5
I	0.5*	11/3/43	18	24	30	35	46	55
	0.5	11/5/43	18	24	30	35	46	56
	0.5	11/5/43	20	30	36	45	62	92
II	0.6*	11/5/43	18	23	30	34	48	56

*Standardized.

plastin was considered to be "standardized" if it gave a normal prothrombin time of from seventeen to nineteen seconds,* if it gave prothrombin times that were comparable† to those obtained with thromboplastins which had been previously used, and if, in addition, it would give a prothrombin time of approximately thirty-five seconds when tested with a 20 per cent dilution of a selected normal plasma. Fortunately there seems to be little change from day to day in thromboplastins prepared as outlined earlier in this paper. Nevertheless, fresh solutions for checking purposes were prepared daily or on alternate days so that at least two thromboplastins giving satisfactory results were available. Often preparations five or six days old checked with the fresh preparations. To say that a single acceptable thromboplastin made up one day should automatically become the standard for comparison the day following might be misleading in some instances. However, the judicious use of the method of checking thromboplastins against each other as outlined has proved to be satisfactory, even though time consuming.

*"Standardized" thromboplastins should give the same prothrombin times with the same normal plasma.

†Not more than 5 per cent variation.

Sometimes it was necessary to reduce the amount of dried brain weighed out in order to obtain a thromboplastin of the desired activity (Table IV).

Thromboplastins of the same concentration (0.8 Gm. in 10 c.c. of saline solution) prepared from different mixtures of dried rabbit brain sometimes showed considerable variation in their activities as evidenced by results obtained on prothrombin-deficient plasmas (Table V). When one thromboplastin was prepared which gave a shorter and another which gave a longer prothrombin time than a "standardized" thromboplastin, a suitable preparation was often obtained by mixing the two in proper proportions (Table VI). This procedure often saved time and material.

TABLE IV

VARIATION BETWEEN TWO DIFFERENT AMOUNTS OF SAME MIXTURE OF RABBIT BRAIN (MIXTURE I) AND PREVIOUSLY STANDARDIZED THROMBOPLASTINS

THROMBOPLASTIN "A" (GRAMS IN 10 C.C. SALINE)	DATE OF PREPARATION	PROTHROMBIN TIME (SECONDS)					
		NORMAL	PATIENTS				
			1	2	3	4	5
0.6*	10/22/43	18	26	31	36	44	55
0.6*	10/22/43	18	27	30	36	45	56
0.6	10/25/43	18	29	35	41	52	66
0.5	10/25/43	18	26	30	35	44	56

*Previously standardized.

TABLE V

COMPARISON OF THROMBOPLASTINS PREPARED SIMULTANEOUSLY FROM FOUR DIFFERENT MIXTURES OF DRIED RABBIT BRAIN*

RABBIT BRAIN MIXTURE	PROTHROMBIN TIME (SECONDS)				
	NORMAL	PATIENTS			
		1	2	3	4
I	18	20	31	42	76
II	18	22	35	44	83
III	19	22	34	46	83
IV	19	23	37	53	102

*Thromboplastin "A," 0.8 Gm. in 10 c.c. of saline solution, was used.

TABLE VI

EFFECT OF MIXING A MORE ACTIVE WITH A LESS ACTIVE PREPARATION OF THROMBOPLASTIN

THROMBOPLASTIN "A"* PREPARATION	PROTHROMBIN TIME (SECONDS)				
	NORMAL	PATIENTS			
		1	2	3	4
I†	18	25	36	54	59
II	18	22	31	44	49
III	18	27	41	63	72
1 part II 1 part III	18	23	33	49	54
1 part II 2 parts III	18	25	35	55	60

*0.6 Gm. in 10 c.c. of saline solution.
†Standardized.

As a plan for testing any thromboplastin of unknown activity it is recommended that thromboplastins be "standardized" to the extent that successively used thromboplastins give the same prothrombin times on the same normal plasmas and, in addition, give comparable prothrombin times on prothrombin-deficient plasmas or give consistent results when tested with a 20 per cent dilution of selected normal plasmas.

Technique of Prothrombin Time Test.—The Quick prothrombin time test, as described by Magath,[23] was used. Whole blood was added to tenth-molar solution of sodium oxalate in the proportion of 9:1. After it had been mixed thoroughly, the blood was allowed to sediment or was centrifuged for a short period at low speed. One-tenth cubic centimeter of plasma was placed in a tube (13 by 100 mm.) and 0.1 c.c. of thromboplastin added to the plasma. The tube with contents was held in a constant temperature bath (37.5° C.). After a few moments, 0.1 c.c. of fortieth-molar solution of calcium chloride anhydrous was added and at the same instant, a stopclock (an electric clock operated by a foot switch has been used to good advantage) was started. The tube was carefully agitated and then tilted at intervals to determine the moment at which a semisolid clot was formed. With thromboplastin "A," the normal prothrombin time was found to be from seventeen to nineteen seconds.

Correlation of Prothrombin Times Obtained With Thromboplastins of Different Activity.—To interpret prothrombin times obtained with a less active thromboplastin in terms of those obtained with a more active thromboplastin presents a problem. In view of this fact it was decided to give some attention to thromboplastins made from acetone-extracted brain. Prothrombin time determinations were performed with thromboplastins (thromboplastin "B") made with varying amounts of the same mixture and with the same amounts of different mixtures of acetone-extracted brain (Tables VII and VIII).

TABLE VII

COMPARISON OF THROMBOPLASTINS PREPARED WITH VARYING AMOUNTS OF THE SAME MIXTURE OF ACETONE-EXTRACTED BRAIN

THROMBOPLASTIN "B" (GRAMS IN 5 C.C. SALINE)	PROTHROMBIN TIME (SECONDS)				
	NORMAL	PATIENTS			
		1	2	3	4
0.2	12	12	17	18	27
0.3	12	12	19	19	30
0.4	13	13	22	22	42
0.5	13	14	24	24	55

TABLE VIII

COMPARISON OF THROMBOPLASTINS PREPARED SIMULTANEOUSLY FROM THE SAME AMOUNT OF FOUR DIFFERENT MIXTURES OF ACETONE-EXTRACTED BRAIN*

RABBIT BRAIN MIXTURE	PROTHROMBIN TIME (SECONDS)				
	NORMAL	PATIENTS			
		1	2	3	4
I	12	17	20	24	27
II	12	18	21	26	30
III	13	20	23	30	39
IV	15	23	27	35	46

*Thromboplastin "B," 0.2 Gm. in 5 c.c. of saline solution, was used.

Prothrombin times were then determined on 100 subjects with three different thromboplastins: thromboplastin "A," average normal, eighteen seconds (prepared according to original method); thromboplastin "B,"* average normal, twelve seconds (prepared according to Quick's modification); thromboplastin "D," average normal, twelve seconds (a commercial preparation†). The correlation between "A" and "B" is shown in Fig. 1; that

*Preparations from selected mixtures of acetone-extracted brain were used; thromboplastins from different mixtures did not always possess similar activity.

†Bacto-thromboplastin supplied through the courtesy of Difco Laboratories, Inc., Detroit, Mich. Thromboplastins of the same lot number apparently possessed similar activity. Some variation existed between thromboplastins from different lot numbers. Further study would have to be made to decide definitely if the variation is significant.

between "A" and "D" in Fig. 2. The prothrombin time which one might expect with thromboplastin "D" if a particular prothrombin time is known for thromboplastin "A" can be obtained from the equation $Y = 2.80 + 0.55\ X$* (Fig. 2). For example, if a prothrombin time of forty-five seconds has been obtained with thromboplastin "A," one would probably obtain a prothrombin time close to twenty-seven seconds if thromboplastin "D" were used. This would hold true of course only if preparations of standard activity were used.

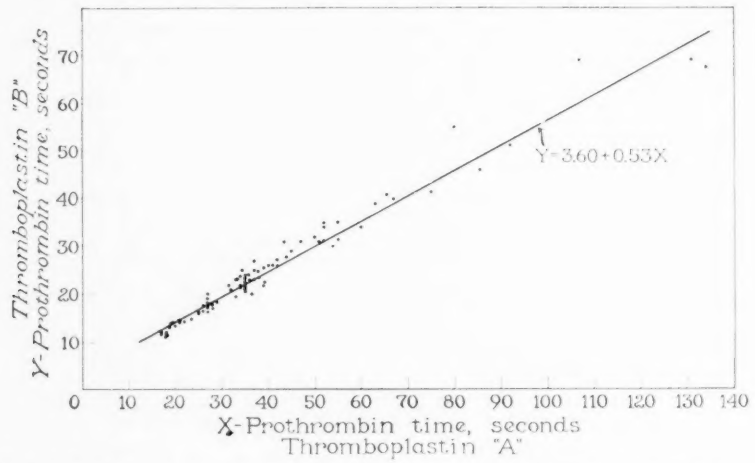

Fig. 1.—Correlation between prothrombin times obtained with thromboplastin "A" and thromboplastin "B."

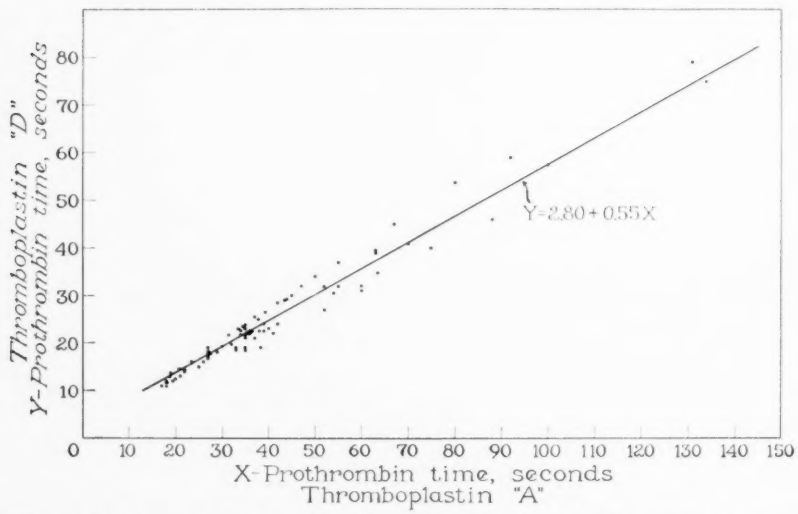

Fig. 2.—Correlation between prothrombin times obtained with thromboplastin "A" and thromboplastin "D."

In addition to this study, prothrombin times with the three different thromboplastins were determined on six patients who were receiving dicumarol. The results with two of the thromboplastins are shown in Fig. 3. Thromboplastin "B" gave results so close to those from thromboplastin "D" that the corresponding curve was omitted.

Prothrombin Times and Prothrombin Percentage.—In an effort to determine the significance of certain prothrombin times in terms of percentage of

*The equation was obtained by the method of least squares by Mr. Robert Gage, of the Division of Biometry and Medical Statistics of the Mayo Clinic.

normal prothrombin, prothrombin time tests were performed on a series of dilutions of ten normal plasmas using thromboplastin "A" and thromboplastin "D." Results are shown in Table IX; an average curve for each thromboplastin is shown in Fig. 4. One cannot say how much prothrombin

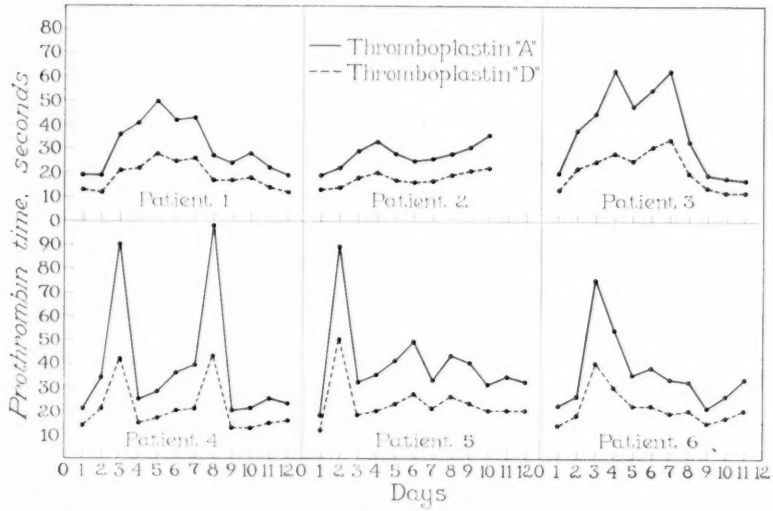

Fig. 3.—Comparison of prothrombin times obtained with thromboplastin "A" and thromboplastin "D" on six patients who were receiving dicumarol.

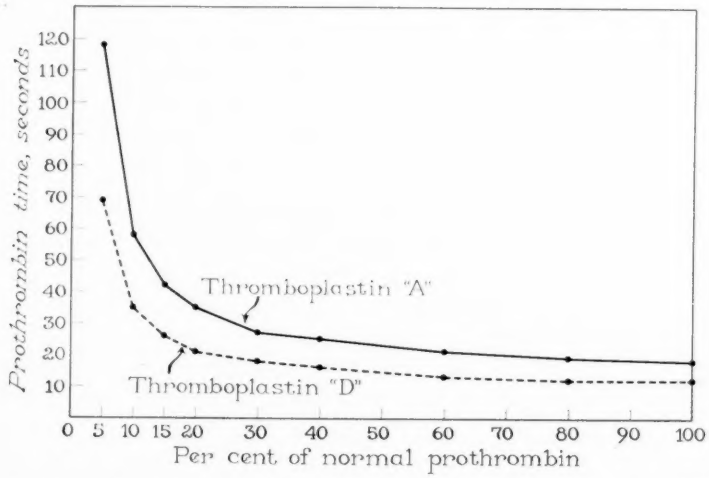

Fig. 4.—Prothrombin dilution curves. Relation between prothrombin time and percentage concentration of normal plasma expressed as per cent of normal prothrombin.

TABLE IX

AVERAGE PROTHROMBIN TIMES ON A SERIES OF DILUTIONS OF TEN NORMAL PLASMAS USING THROMBOPLASTINS "A" AND "D"

PER CENT NORMAL PLASMA	PROTHROMBIN TIME (SECONDS)	
	THROMBOPLASTIN "A"	THROMBOPLASTIN "D"
100	18	12
80	19	12
60	21	13
40	25	16
30	27	18
20	35	21
15	42	26
10	58	35
5	118	69

is present in normal plasma, but for purposes of comparison the original plasma without further dilution was considered to have 100 per cent prothrombin and each normal plasma was so diluted that it was considered equivalent to 80, 60, 40, 30, 20, 15, 10, and 5 per cent of normal prothrombin. Dilutions were made with prothrombin-free plasma, prepared according to the Seitz method of Kark and Lozner.[21] When physiologic saline solution was used as a diluent, end points were difficult to determine when the concentration of plasma was less than 20 per cent. Prothrombin times determined on diluted plasmas, when concentrations were 20 per cent or more, were essentially the same with either diluent when thromboplastin "A" was used. With thromboplastin "D," prothrombin times on diluted plasmas were somewhat prolonged when saline solution was used. Some investigators[20, 36] have recommended the use of fibrinogen solution as a diluent in the preparation of diluted plasmas.

When one applies prothrombin times from the appropriate curve in Fig. 4 to the equation, $Y = 2.80 + 0.55X$ (Fig. 2), it will be seen that the calculated prothrombin times for the various dilutions closely check the observed prothrombin times for the same dilutions. Theoretically and by actual experiment, twenty-seven, thirty-five, and sixty seconds (actually fifty-eight seconds) obtained with thromboplastin "A" become equivalent to eighteen, twenty-one, and thirty-five seconds with thromboplastin "D." Again, this correlation holds true only when selected or "standardized" thromboplastins are used. Though one hesitates to say that when a particular prothrombin time is equivalent to that obtained with a 20 per cent dilution of normal plasma, the plasma being tested therefore contains 20 per cent of prothrombin, yet for purposes of comparison, dilution experiments provide certain information. Dilution may have an effect on certain coagulation factors. It is thought, for example, that dilution reduces the effect of clotting inhibitors.

Critical Levels of Prothrombin.—Prothrombin times of twenty-seven seconds and sixty seconds indicate critical levels of prothrombin when thromboplastin "A" has been used in the prothrombin time test. A prothrombin time of thirty-five seconds (thromboplastin "A") has been used as a guide to dosage of dicumarol. Because of the difficulty of comparing these prothrombin times with those considered significant when a thromboplastin of different activity has been used, it has seemed advisable to express twenty-seven, thirty-five, and sixty seconds in percentage of normal prothrombin. On examination of the appropriate curve in Fig. 4, it will be noted that, when thromboplastin "A" is used, these prothrombin times of twenty-seven, thirty-five, and sixty seconds correspond to the prothrombin times obtained with 30, 20, and 10 per cent normal plasma, respectively. Prothrombin times of eighteen, twenty-one, and thirty-five seconds were obtained on these same dilutions when thromboplastin "D" was employed. Since prothrombin times of twenty-seven, thirty-five, and sixty seconds (thromboplastin "A") corresponded to eighteen, twenty-one, and thirty-five seconds (thromboplastin "D") when original plasmas were tested (Fig. 2), it seems to justify further this method of comparing prothrombin times obtained with two different thromboplastins.

If one is using a thromboplastic substance of different or unknown potency and one wishes to select a prothrombin time equivalent to the thirty-five seconds obtained with thromboplastin "A," the most satisfactory method would be to determine the prothrombin times on 20 per cent dilutions of ten normal plasmas. The average of these determinations would give a prothrombin time which could serve as a guide to dicumarol dosage as outlined by Allen, Barker,

and Waugh.[2] Prothrombin times indicative of 30 and 10 per cent levels of normal prothrombin could be ascertained by determining the prothrombin times on the corresponding dilutions. Comparison of prothrombin times obtained with a 20 per cent dilution of normal plasma with those obtained with a prothrombin-deficient plasma containing approximately 20 per cent prothrombin is shown in Tables X and XI. Thromboplastins of different activity were employed.

TABLE X

COMPARISON OF PROTHROMBIN TIMES ON A 20 PER CENT DILUTION OF NORMAL PLASMA WITH THOSE OBTAINED ON A PROTHROMBIN-DEFICIENT PLASMA USING THROMBOPLASTIN "A"

PLASMA		PROTHROMBIN TIME (SECONDS)				
		I*	II	III	IV	V
Normal	Original	18	18	18	18	18
	20 per cent dilution	35	38	41	41	43
Prothrombin-deficient†	Original	34	36	38	39	40

*Preparations of thromboplastin "A" of different activities.
†Patient receiving dicumarol.

TABLE XI

COMPARISON OF PROTHROMBIN TIMES ON A 20 PER CENT DILUTION OF NORMAL PLASMA WITH THOSE OBTAINED ON A PROTHROMBIN-DEFICIENT PLASMA USING THROMBOPLASTIN "B"

PLASMA		PROTHROMBIN TIME (SECONDS)				
		I*	II	III	IV	V
Normal	Original	12	13	15	16	17
	20 per cent dilution	22	26	30	35	35
Prothrombin-deficient†	Original	21	25	29	35	36

*Preparations of thromboplastin "B" of different activities.
†Patient receiving dicumarol.

Dilution of Normal Plasma as a Means of Evaluating the Activity of Thromboplastin.—A dilution curve of normal plasma has been used by numerous workers to determine the percentage of prothrombin present in the blood. Since the curve obtained depends in part on the thromboplastin employed, it may well be used as a method of evaluating the activity of a particular thromboplastic substance. As a routine procedure the establishment of two or three selected points on the curve would probably give the necessary information. Dilutions should be made from a selected normal plasma (one previously studied) or from a sufficient number of normal plasmas to obtain what seems to be a representative curve. When the activities of different preparations of thromboplastin, prepared simultaneously, are being compared, the method of choice is to use dilutions of the same plasma. Some apparently normal plasmas may give curves at somewhat different levels. For this reason the use of selected plasmas will minimize the influence of other coagulation factors on the coagulation time obtained.

COMMENT

Some comment is probably in order concerning the continued use of Quick's original method. When the modification was first proposed by Quick, it was realized that for the clinician to interpret results which would be obtained in terms of those with which he was already familiar would involve performance of both methods for some time. Furthermore, the possible advantages did not seem to outweigh certain disadvantages, the chief one being that such an active preparation could hardly show actual differences in prothrombin content that

would be apparent with a less active preparation. Souter and Kark,[35] in 1940, made a similar observation and stated that "in a test such as this when the time of the reaction represents the concentration of one interacting substance, it is essential for accurate estimation of the limits of that substance present that the decrease in its concentration should be reflected by an appreciable prolongation of the time of the reaction."

With the advent of dicumarol therapy, the problem was reconsidered. In addition to the conclusion drawn previously, which seemed to apply now more than ever, it was felt that the use of acetone-extracted brain did not assure more consistent results than when brain not extracted with acetone was used. True, the number of seconds of variation was usually less, but when one considered the change in prothrombin percentage or activity presented by the difference in time, interpretation of the results became more difficult. Thromboplastins prepared from different mixtures of acetone-extracted brain showed greater variation in their activities than did those prepared from the same mixture. This was partly due to the manner in which the brain was extracted with acetone. Standardization of details in the method of extraction eliminated marked differences in the normal prothrombin times but did not eliminate significant differences when prothrombin times were prolonged.

Quick[31] has stated that, because of the explicit directions which he has given for the preparation of a highly active thromboplastin, there is no longer valid excuse for reporting prothrombin data based on thromboplastin which does not give a normal prothrombin time of twelve and one-half seconds. If one assumes that this be true, the fact still remains that the use of such a thromboplastin would not necessarily assure consistent results on prothrombin-deficient* plasmas. This, after all, is the real test of thromboplastic activity. It may seem that greater variation than 5 per cent should be allowed in prothrombin times obtained with prothrombin-deficient plasmas. But since thromboplastins are frequently prepared which check almost to the second when the prothrombin percentage is between 5 and 10 per cent, one cannot help feeling that when it is more than 10 per cent, differences in prothrombin time should represent differences in prothrombin and not differences in thromboplastic activity. Perhaps one is not justified in spending so much time to obtain consistent results with a test which is dependent on so many variables. But because prothrombin times thus obtained have been of great value from a clinical standpoint, one hesitates to depart from this established system of "standardization." Some state that they do not find variations in thromboplastins as prepared by them. However, since others do find such variation, the problem cannot be ignored.

When one considers the complexity of the factor or factors possessing thromboplastic activity, which are present in tissue, it is hardly possible that all preparations of any tissue extract should be equally potent. The presence of clotting inhibitors in tissue has been demonstrated.[6] Clotting inhibitors as well as clotting activators may be responsible for some of the variation which occurs in the activity of tissue extracts.

Souter and Kark[35] have recommended the preparation of stable thromboplastin by utilizing the method of "lyophilization" introduced by Flosdorf and Mudd.[17] This is a complicated procedure not easily adapted to a clinical laboratory. While it would have certain advantages over other procedures if it could be simplified, it would seem that some standardization as to activity should be made before the thromboplastin is lyophilized.

*In this study, the expression "prothrombin-deficient" is used to designate those plasmas giving prolonged prothrombin times.

As has been shown, reducing the concentration of a tissue extract, under certain conditions, increases its activity. A similar observation was made by Aggeler and Lucia[1] and by Kazal and Arnow,[22] who noted that dilution of a thromboplastin increased its activity. It is possible that making serial dilutions of a particular thromboplastin might enable one to select a preparation of the desired activity.

Though prothrombin time tests using Russell viper venom have been performed in this study, comment concerning the results will not be made at this time. Numerous workers[19, 26, 33, 39, 40] have substituted venom for tissue extract in the Quick prothrombin time test. Worthy of mention is the difference in prothrombin time obtained with venom alone and venom plus lecithin. Witts and Hobson[39] have termed this the "lecithin difference." Witts[38] has referred to the influence of platelets on the effect of Russell viper venom, an influence which was comparable to that of lecithin.

Some investigators have followed the modification by Link and his associates[5] of Quick's test by determining the prothrombin time on diluted plasma in order to measure the effect of dicumarol. This has not seemed necessary as a routine procedure, at least when the less active thromboplastin (thromboplastin "A") was used. Whether the use of a diluted plasma in addition to undiluted plasma does furnish more accurate information in specific instances, as to the amount of prothrombin contained in the plasma,[33] is being studied further.

Since plasma dilution curves may be influenced by variables, known and unknown, it is well to keep in mind that a plasma giving a prothrombin time equivalent to that obtained with a 20 per cent dilution of normal plasma may not actually contain 20 per cent of normal prothrombin. However, the dilution curve method would seem to be the best method at our disposal to obtain an expression of prothrombin times in terms of prothrombin percentages. If the two-stage method of determining prothrombin were more applicable to clinical problems, the simultaneous performance of the two methods might furnish valuable information.

In particular, the dilution curve of normal plasma gives important information as to the activity of the thromboplastin employed. This is true, not only when comparing the activity of one thromboplastin with that of another,* but especially when establishing critical levels of prothrombin deficiency. Since prothrombin time with any given method is determined not only by prothrombin content, but also by thromboplastic activity, the dilution curve best depicts the influence of these two coagulation factors. When thromboplastin "A" was used, it has been shown that the critical prothrombin times of twenty-seven, thirty-five, and sixty seconds might preferably be expressed as 30, 20, and 10 per cent of normal prothrombin. For purposes of comparison, prothrombin percentages† are more easily evaluated than are prothrombin times in seconds. It is absolutely essential, however, that each investigator establish a normal dilution curve for the method as performed. Prothrombin times should not be expressed as prothrombin percentage on the basis of a dilution curve obtained by someone else. In some instances dilution curves obtained by different investigators may be the same, but this is not necessarily so.

*Nygaard[25] found that the correlation of prothrombin time in seconds and varying concentrations of a thromboplastin emulsion presented a better check as to potency than could be obtained by a single observation.

†Some ambiguity of expression exists when the ratio of a particular prothrombin time to the normal prothrombin time is expressed as per cent of normal or per cent prothrombin. Thirty-five seconds may denote a clotting activity equal to 51 per cent of that indicated by a normal time of eighteen seconds, but it is equivalent only to 20 per cent of normal prothrombin concentration. The relationship between prothrombin time and prothrombin concentration is not linear.

Hoffman and Custer[20] stated that "thromboplastin of optimal potency has probably not yet been obtained and many reports have appeared indicating that the standard and near optimal potency described by Quick was not used. When thromboplastin becomes a limiting factor, absolute values are no longer comparable and relative ones (i. e., per cent of normal) have not been shown to be strictly so."

In this study considerable emphasis has been placed on the factors, thromboplastin in particular, which may influence the prothrombin times obtained with the Quick prothrombin time test. However, this in no way is meant to minimize the importance of the Quick prothrombin time test, a test which continues to be the indispensable guide to dicumarol therapy.

SUMMARY

The Quick prothrombin time test is a reliable guide to the administration of dicumarol, provided thromboplastins of constant potency are used.

Prothrombin time tests have been performed simultaneously using a less active thromboplastin (thromboplastin "A," average normal, eighteen seconds) and more active thromboplastins (thromboplastins "B" and "D," average normal, twelve seconds).

Prothrombin times, indicative of critical levels of prothrombin, expressed as prothrombin percentages enable one to compare results obtained with thromboplastins of different activities.

The important levels of prothrombin deficiency during dicumarol therapy are: the level below which thrombosis is unlikely to occur, the level which serves as a guide to dicumarol dosage, and the level above which bleeding is unlikely to occur.

With thromboplastin "A" these levels have been established as 30 per cent (twenty-seven seconds), 20 per cent (thirty-five seconds), and 10 per cent (sixty seconds), respectively.

With thromboplastins "B" and "D" the prothrombin times denoting these critical levels have been established as eighteen seconds (30 per cent), twenty-one seconds (20 per cent), and thirty-five seconds (10 per cent).

Prothrombin times denoting critical levels when still other thromboplastins are used may be ascertained by determining the prothrombin times on appropriately diluted normal plasmas.

The activity of a particular thromboplastin may be evaluated by determining the prothrombin times on a series of dilutions of normal plasma.

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PROTHROMBIN STUDIES USING RUSSELL'S VIPER VENOM

VII. Effect of Prothrombin Clotting Time of the Concentrations of Calcium and Other Salts

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WHEN rabbit brain extract is used as a thromboplastic substance in the determination of prothrombin clotting time, the time required for clot formation is prolonged if the concentration of the calcium salt is increased or if other salts are added.[1-4]

The present investigation was conducted to determine whether, when Russell's viper venom[5, 6] was employed as a thromboplastic substance, the prothrombin clotting time was similarly affected by calcium and other salt concentrations, and to compare the two thromboplastic materials under similar conditions of calcium and other salt concentrations. For partial substitution for the calcium, magnesium chloride was selected as a representative of divalent ions and sodium chloride of monovalent ions.

Method.—Plasma was prepared from blood samples obtained by venipuncture, 9 c.c. of blood being put into a test tube containing 20 mg. of potassium oxalate. After mixing and centrifugalizing, the plasma was drawn off. Each test was completed within one hour after preparation of the plasma.

Two-tenths cubic centimeter of plasma was pipetted into a small test tube and then 0.2 c.c. of thromboplastin extract* was added and brought to 36 to 38° C. in a water bath. Two-tenths cubic centimeter of the particular salt solution was added and a stop watch was started. The tube was then gently rocked until the first appearance of a clot was noted. This time was recorded as the prothrombin clotting time. The type of clot formed by the venom solution differs from that which appears with the rabbit brain extract. With brain extract the clot forms a soft gelatinous mass, the time of the first appearance being taken as the end point. With viper venom the clot begins as fibrin particles with subsequent formation of a solid gel; the first formation of fibrin particles is taken as the end point.

Preparation of Salt Solutions.—Two-tenths molar calcium chloride was prepared by adding a slight excess of calcium carbonate (reagent grade) to an accurately measured amount of 1.0 M hydrochloric acid in a volumetric flask, heating the flask and contents in a boiling water bath with frequent shaking for twenty minutes, cooling, making up to volume with distilled water, and filtering to remove the slight excess of carbonate. Lower concentrations were prepared by dilutions with distilled water. This method was employed because of the difficulty encountered in the direct weighing of calcium chloride, due to its uncertain composition and hygroscopic property.

Two-tenths molar magnesium chloride solution was prepared in a similar manner by adding an excess of magnesium oxide to a measured amount of 1.0

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*Rabbit brain extract used in this investigation was prepared from the dry powder, "Bactothromboplastin," Difco Laboratories, Inc., Detroit, Mich. Russell's viper venom, "Stypven," was obtained from Burroughs Wellcome and Co., New York, N. Y.

molar hydrochloric acid in a volumetric flask, heating in a water bath for twenty minutes, cooling, making up to volume, and removing the excess oxide by filtration.

Sodium chloride solution, 1.0 molar, was prepared by dissolving a weighed amount of the salt in distilled water.

DISCUSSION

In the first series, consisting of seventeen patients, both the viper venom and the rabbit brain extract methods were studied, with the calcium chloride concentration alone being varied.

In the second series, consisting of eighteen patients, the calcium was partially replaced by magnesium or by sodium; a constant concentration of 0.025 molar calcium chloride was used, with increasing amounts of magnesium chloride and sodium chloride being added. The results are shown in Table I as the averages. Standard deviations are also indicated.

The results show that when viper venom was used, the prothrombin clotting time was prolonged as the concentration of calcium chloride was increased; however, to a lesser extent than when the rabbit brain extract is used. The substitution of other salts for some of the calcium produced a similar result. The difference routinely noted between the rabbit brain "average normal" prothrombin clotting time of about fifteen seconds and the viper venom of twenty seconds is apparently due to the lower calcium chloride concentration, M/40 used in the former as compared with M/10 used in the latter in standard laboratory routine. When M/40 calcium chloride is used in the viper venom method, the average normal time is fifteen seconds.

TABLE I

EFFECT OF CONCENTRATION OF CALCIUM CHLORIDE AND OF PARTIAL REPLACEMENT OF CALCIUM CHLORIDE BY MAGNESIUM CHLORIDE AND BY SODIUM CHLORIDE ON PROTHROMBIN CLOTTING TIME

CONCENTRATION OF SALT			RUSSELL'S VIPER VENOM			RABBIT BRAIN THROMBOPLASTIN		
$CaCl_2$	$MgCl_2$	NaCl	MEAN VALUE (SEC.)	STANDARD DEV.	STANDARD ERROR OF MEANS	MEAN VALUE (SEC.)	STANDARD DEV.	STANDARD ERROR OF MEANS
First Series								
0.025M			15.5	2.4	0.6	15.0	1.1	0.3
0.050			16.1	2.4	0.6	20.6	1.9	0.5
0.10			20.4	3.2	0.7	47.7	2.6	0.6
0.20			46.3	9.6	2.3	201.5	46.8	11.4
Second Series								
0.025			14.5	2.5	0.6	14.6	1.1	0.2
0.025	0.025		15.4	2.3	0.5	17.1	1.4	0.3
0.025	0.075		24.1	4.6	1.1	32.2	5.6	1.3
0.025	0.175		105.3	24.4	5.7	157.6	34.7	8.2
0.025		0.050	15.3	2.5	0.6	15.1	2.2	0.5
0.025		0.150	16.4	2.2	0.5	16.3	1.9	0.5
0.025		0.350	23.3	3.2	0.8	22.1	3.6	0.9

A practical aspect of the less pronounced effect of calcium concentration in the viper venom method is that it allows for greater flexibility of the entire procedure. The concentration of the calcium chloride solution need not be exact but may be somewhat less than 0.1 M and the quantity of oxalate used as anticoagulant and the relative volume of the blood sample need only be approximate; thus the usual routine methods are adequate and special collection technique is not required.

SUMMARY

Increased calcium chloride concentration prolongs the prothrombin clotting time when Russell's viper venom is used as the thromboplastic substance. The effect is not as pronounced as with rabbit brain extract.

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FATAL CARDIAC TAMPONADE FOLLOWING STERNAL PUNCTURE

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DEATH from cardiac tamponade is a complication which may follow sternal puncture. The widespread use of the operation as a supposedly innocuous procedure prompts this report of a second death from sternal puncture and the first to come to autopsy.

CASE REPORT

E. S., a white male farmer 31 years of age, was admitted to the Medical College of Virginia Hospital April 30, 1943, for diagnosis. His chief complaint was pain in the left leg and weakness.

Four months previously the patient had noticed pain in the back which radiated over the kidney region. He continued at work, the pain being intermittent and usually associated with effort. Later he noticed sharp pain in the left chest over the precordium which also was intermittent. Because of this pain he consulted his physician who referred him on April 24, 1944, to a local hospital where x-ray studies revealed a left mediastinal mass thought to be an enlarged pulmonary conus. He was referred to the Medical College of Virginia Hospital. During the several intervening days the small toe on his left foot became sore and swollen. This was followed on the day previous to admission by a redness of the foot and leg which had gradually increased until it reached the inguinal region. On his way to this hospital by automobile he noticed the appearance of purpuric spots on his legs and arms.

The past history was essentially negative except for the somewhat frequent occurrence of epistaxis during the past four months. There had been a loss of about twelve pounds in weight.

Admission examination revealed an acutely ill, well-developed man with a temperature of 104° F. The nose was bleeding and filled with blood clot. There was a diffuse fine purpuric spotting of the body involving especially the arms and legs. Examination of the heart and lungs proved negative, blood pressure, 160/80 mm. Hg. Although the abdomen was distended with gas, the spleen was palpable. The small toe of the left foot was red and swollen, and red streaks followed the medial side of the leg to the inguinal region where the inguinal glands were palpable. These were the essential findings.

From the Departments of Medicine and Pathology, Medical College of Virginia.
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Laboratory examination by the attending intern revealed: red blood cells, 4,370,000 per cubic millimeter; hemoglobin, 7.3 Gm. per 100 c.c.; white blood cells, 8,850, 80 per cent polymorphonuclears and 20 per cent lymphocytes; platelets, 140,000 (normal, 500,000). Urinalysis, negative. Roentgenogram of the chest showed a mediastinal mass which was thought to be a prominent pulmonary conus.

The leg was placed in wet dressings and sulfathiazole was administered. Transfusion was given the evening of admission and repeated May 2 and 3 as the epistaxis continued. On this last date the following blood count was reported by the intern assigned to the case: red blood cells, 2,640,000; hemoglobin, 7.3 Gm.; white blood cells, 9,000, 77 per cent polymorphonuclears, 2 per cent eosinophiles, 18 per cent lymphocytes, 2 per cent monocytes. A check of this count by the laboratory showed 2,400,000 red cells, 38,400 platelets, and normal prothrombin time, bleeding time, and coagulation time. Clot retraction was good in fifteen hours.

With a tentative diagnosis of purpura or leucemia, a blood film was submitted to one of us (J. H. S.). Study of the preparation resulted in the following report: "Essentially normal-appearing red cells with a marked lack of platelets, frequent myelocytes, and promyelocytes. While this could be leucemia, I would be more inclined to favor a metastatic carcinoma with a myelophthisic anemia and thrombocytopenia."

Several unsuccessful attempts were made by the intern staff to aspirate marrow from the sternum. Following these failures, one of us (J. H. S.) was requested on May 4 to perform sternal puncture. With an 18-gauge spinal needle cut off to a length of about 1¼ inches, several attempts were made to reach the marrow cavity of the sternum at a level between the third and fourth ribs. The depth to which the needle was inserted at times reached about three-fourths of an inch. Repeated attempts at several levels resulted only in the aspiration of a small amount of blood-tinged fluid. The procedure was given up as unsuccessful. Shortly thereafter, the intern, about to start a blood transfusion, found the patient pale, semicomatose, breathing noisily, and with pupils dilated. Pulse was thready and rapid and blood pressure not determinable. The foot of the bed was elevated, coramine and adrenalin given, and artificial respiration started. The patient died suddenly after a few minutes, approximately thirty minutes after the unsuccessful aspiration.

Autopsy revealed the following significant findings:

Thorax: There was a normal disposition of thoracic visceral structures. There was a through and through needle puncture wound of the sternum just below the sternal angle with slight periosteal extravasation of blood about the point of exit on the posterior surface of the sternum. Directly beneath this wound there was a similar needle puncture wound through the parietal pericardium. The pericardial sac was distended by approximately 350 c.c. of fresh blood and the heart was completely surrounded by fresh clot. Just beneath the puncture wound of the parietal pericardium there was a triangular laceration of the musculature of the right ventricle 1 cm. in greatest diameter. The pleural cavities contained no free fluid and had smooth, glistening serosal surfaces. Both lungs were moderately collapsed.

Tumor: There was a firm, rounded mass 4 by 6 cm. in diameter located in the anterior mediastinum overlying the pulmonary conus. This mass was not a part of any mediastinal structure and was connected to the adjacent structures only by the loose connective tissue of the mediastinum. Section of the mass revealed a definite fibrous capsule enclosing a hemorrhagic parenchyma, composed of tissue strands separating small cystic spaces, some of which contained clotted blood. One pole contained an irregular cavity several centimeters in diameter with a smooth lining from which several nodules projected into the cyst. No cartilage, bone, or other mesodermal structures could be detected in this mass grossly. Mediastinal visceral structures otherwise showed normal development, position, and relationships.

Heart: The heart weighed 400 grams. It was of normal contour and showed the usual size relationship between the right and left ventricles. The epicardial surface was smooth and glistening and showed a few scattered petechiae. There was a 1 cm. triangular laceration of the musculature of the anterior surface of the right ventricle, 3 cm. proximal to the origin of the pulmonary conus. The laceration was 5 mm. deep but did not communicate with the cavity of the right ventricle, being separated by intact endocardium and 1 or 2 mm. of intact myocardium. There was no injury to any major branch of the coronary arteries. The myocardium was uniformly reddish-brown in color and of firm consistency. The usual size differential in the thickness of the right and left ventricular walls was present. The endocardium was smooth and glistening except for a few petechiae. All valves were smooth, resilient, and anatomically competent. The chordae tendineae were not thickened or shortened.

The coronary ostia were wide and the coronary arteries of normal caliber throughout with smooth glistening intimae. The ascending aorta was of uniform diameter with thin, resilient walls and smooth intimal surface. There were a few minimal atheromatous deposits in the intima. There was a normal configuration of the major vessels arising from the arch.

Lungs: The left lung weighed 300 grams and the right 350 grams. Both lungs were uniformly fairly well collapsed. The external surfaces of all lobes were grayish-pink except for a well-outlined portion of both lower lobes which was dark blue. The visceral pleura, especially on the interlobar surfaces, showed fairly numerous petechiae. Sections through both lower lobes showed a deep red noncrepitant area which was sharply demarcated from the normal-appearing parenchyma. There were no focal lesions and no increased fluid present in this collapsed area. The bronchi of both lungs were largely filled with bloody mucopurulent material but contained no actual clotted blood.

Spleen: The spleen weighed 400 grams. It showed approximately normal contour with some notching of the anterior border. The external surface was reddish-blue in color with a thin, smooth capsule. The consistency was moderately soft. Section showed a dark red friable parenchyma in which a few small follicles were visible.

Kidneys: The left kidney weighed 250 grams and the right 250 grams. Both were of normal size and shape and were firm in consistency, the capsule strips readily leaving a smooth, reddish-brown surface. Section showed a normal configuration of medulla and cortex with prominent cortical striations. The right renal pelvis contained clotted blood. Both ureters were normal in caliber and position and contained no clotted blood.

Liver: The liver weighed 2,900 grams. It was moderately enlarged and showed smooth rounded contours and normal lobar configuration. The consistency was moderately firm and the color reddish-brown. The lobular markings were distinct.

The pertinent microscopic findings were as follows:

Tumor: Numerous sections from various areas of the mediastinal tumor showed a varied appearance. There was very extensive necrosis occupying two-thirds of the total tumor tissue. In the better-preserved areas there was irregular arrangement of varied tissues including glands of various types, nests of squamous epithelium, myxomatous tissue, and cartilage. In some areas the appearance was that of a highly malignant tumor but there was marked variation in the picture from one area to another. In other areas the picture was that of fibrosarcoma or reticulum cell sarcoma. In still other areas there were definite nests of anaplastic epithelial cells and glands, while still other areas showed cells of two different types, large and small, in irregular arrangement, suggesting seminoma. Occasional areas showed infiltration by small round and plasma cells.

Mediastinal Lymph Nodes: In one section the subcapsular sinuses were filled with large tumor cells. These occurred as individual cells and the lymph node structure was not disturbed. Another section showed widespread infiltration of the sinuses by similar tumor cells but again the lymph node structure was not destroyed.

Bone Marrow: Sections from sternum and ribs showed almost complete replacement of normal marrow by anaplastic tumor tissue containing numerous giant cells and many mitotic figures. There was very extensive necrosis of the tumor and marrow with fairly marked destruction and fibrous tissue replacement of the marrow cells.

Spleen: The spleen was very cellular with prominent follicles. Anaplastic tumor cells were present throughout the pulp and sinuses, both diffusely and in groups. Many of them were giant cell forms with several nuclei, while mitotic figures and partially necrotic cells were plentiful. There were immature myeloid cells, a number of them eosinophilic myelocytes.

Liver: The liver cells had a foamy appearance due to glycogen. The sinusoids contained numerous immature cells, many showing mitotic figures. Some of these resembled blast forms of the myeloid series; others were apparently tumor cells. There was no leucemic infiltration or areas of hemopoiesis.

The final anatomical diagnoses were as follows:

1. Perforating needle wound of sternum and pericardial sac.
2. Laceration of anterior wall of right ventricle with hemopericardium and cardiac tamponade.
3. Malignant teratoma of anterior mediastinum with metastases to mediastinal lymph nodes, bone marrow of sternum and ribs, spleen, and sinusoids of liver.
4. Splenomegaly with myeloid metaplasia.
5. Hepatomegaly.
6. Petechial hemorrhages of skin and massive hemorrhage into renal pelves.

7. Partial atelectasis of both lower pulmonary lobes.
8. Superficial ulceration of left small toe.

DISCUSSION

Meyer and Halpein* reported the first death from sternal puncture. As in our case, they experienced difficulty in aspirating marrow, and their patient died shortly thereafter with feeble, rapid pulse, unobtainable blood pressure, and distended neck veins. Their patient experienced no pain other than that which might be expected in an individual made more apprehensive by unsuccessful attempts at aspiration. The same may be said of our patient. Unable to secure autopsy, Meyer and Halpein attributed death to "cardiac inhibitory reflexes through the vagus, initiated by fear." As the signs in the two cases were identical, it seems not unlikely that their patient also died of cardiac puncture and tamponade. Cardiac tamponade is accompanied by pallor with distended neck veins, while on the other hand vasovagal syncope (shock) is accompanied by pallor but with completely collapsed veins.

In a large series extending back to 1936, one of us (J. H. S.) has experienced no other accident or sequela of any type in performing sternal puncture. With the exception of the first year or two, he has used an 18-gauge spinal needle sawed off and rebevelled to a length of about 1¼ inches and has employed the technique of boring directly down into the mid-sternum at the level of the third or fourth rib. There have been occasional difficulties in finding and aspirating marrow. Such cases have usually proved to be patients with osteosclerosis, sclerosing metastatic tumors, or Paget's disease. Under such circumstances, the operator has often felt that he had pierced the lower sternal plate, but no untoward effects followed.

The case under discussion was one of metastatic tumor producing considerable fibrosis in the marrow. This seems sufficient to account for the difficulty encountered. The fact that the needle reached the heart may have been due to altered mediastinal conditions, the large tumor possibly forcing the heart nearer the sternum than normal.

It might be expected that puncture of the heart, even with an 18-gauge needle, would not result in fatal hemorrhage. However, the needle was fixed in the sternum and the point embedded in the wall of the right ventricle, which apparently resulted in laceration of the myocardium due to the movement of the heart against a fixed, sharp object, the needle point. It is interesting that the laceration did not communicate with the ventricular cavity or with any major branch of the coronary arteries. Apparently, hemorrhage from the myocardium was sufficient to produce fatal tamponade within the course of thirty minutes.

Since this unfortunate accident, whenever difficulty has been experienced, the needle has been withdrawn and a different technique followed. This consists of inserting the needle into the sternum in the same area from below at an angle of 45 degrees. In our experience, whenever difficulty is encountered, the bone has a gritty, porous, corklike feel which makes it easy to penetrate and difficult to determine position. At such an angle, the danger of deep penetration is reduced to a minimum.

SUMMARY

The second death from sternal puncture recorded in the literature and the first autopsy on such a case is reported. The cause of death was cardiac tamponade, due to hemorrhage from laceration of the right ventricle by the needle.

*Meyer, Leo M., and Halpein, Jacob: Death Following Sternal Puncture, Am. J. Clin. Path. 14: 247, 1944.

AN INTERMEDIATE TYPE OF OVALOCYTOSIS IN A NEGRO

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AN INCREASE in number of oval or elliptical erythrocytes above the Gunther index[4] in healthy individuals is commonly referred to as "ovalocytosis." This phenomenon is considered a nonsex-linked Mendelian dominant character occurring in the white and the Negro races. According to Florman and Wintrobe,[3] these ovalocytes or ellipsoid cells do not undergo a change in shape when subjected to the sealed chamber methods. Hunter and Adams[5] claim that at 54° C. the elliptical cells become discoid in shape. Terry and coworkers[9] and Cheney[1] noted that repeated washing in isotonic saline caused a change of the ovalocytes to round forms. Leitner,[6] according to Wintrobe,[10] found no abnormality in resistance to hypotonic saline, although Stephens and Tatelbaum[8] noted the presence of poikilocytes. Miller and Lucas[7] stress that the elliptical erythrocyte is not a poikilocyte but a congenital anomaly compatible with health. Dameshek[2] described a condition under the name of "Mediterranean Target-Oval Cells Syndrome" in which ovalocytes are part of the peripheral blood pattern. He observed an increased resistance of the erythrocytes to hypotonic saline.

It is the purpose of this paper to report a series of experiments done on a fresh sample of blood obtained by venepuncture from a healthy adult Negro whose erythrocytes were distinctly elliptical (Fig. 1) but which rounded up under sealed conditions (Fig. 2). This change of shape could be reversed. It is assumed that the observations reported here suggest the existence of an, as yet, little investigated "intermediate type of ovalocytosis" belonging in the group of "erythrocyte thinness." There were 86 elliptical cells per 100 erythrocytes, few macrocytes and microcytes, and occasionally a red cell believed to be a poikilocyte. A sealed preparation was made; after fifty-six hours, no sickle cells were present, but practically all elliptical erythrocytes were rounded up. A small sample of blood was repeatedly washed in physiologic saline solution and some rounding up in shape was noted. These washed cells were submitted to the seal test. One preparation was placed in the icebox and another exposed to room temperature. In both preparations the cells had completely rounded up after twenty-four hours. Addition of the donor's plasma to these twenty-four hour preparations caused the cells to assume their former elliptical shape. No increase of poikilocytes was observed. Another sample of washed cells was sealed until rounded up while exposed to room temperature. The seal was then broken and the rounded up erythrocytes returned to their original shape after five hours. Fragility tests on the stock sample and of the washed cells were essentially normal. To a sample of washed and rounded up cells an equal amount of plasma of the same blood type was added; within a few hours the erythrocytes assumed their elliptical shape. A sample of the original blood was permitted to remain in the donor's plasma, and the preparation was stored in the refrigerator for eight days. The majority of the elliptocytes retained their original shape.

The donor's serology was negative. A history of tuberculosis could not be obtained. The blood icteric index was 5 units. Reticulocytes were 1 per cent.

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There was no anemic state. Mean corpuscular diameter of the erythrocytes was 7.7 microns.

DISCUSSION AND SUMMARY

The observations presented in this paper seem to suggest that this apparent biologic variable of true ovalocytosis is of an intermediate type and does not necessarily represent a pathologic state. As far as could be determined, this Negro donor enjoyed good health at the time the blood sample was obtained. His erythrocytes were not only distinctly elliptical, but the cells rounded up in the sealed chamber after twenty-four to fifty-six hours. This observation does not support the finding of Florman and Wintrobe, that in the true ovalocytosis the erythrocytes retain their original shape. The change in form would suggest

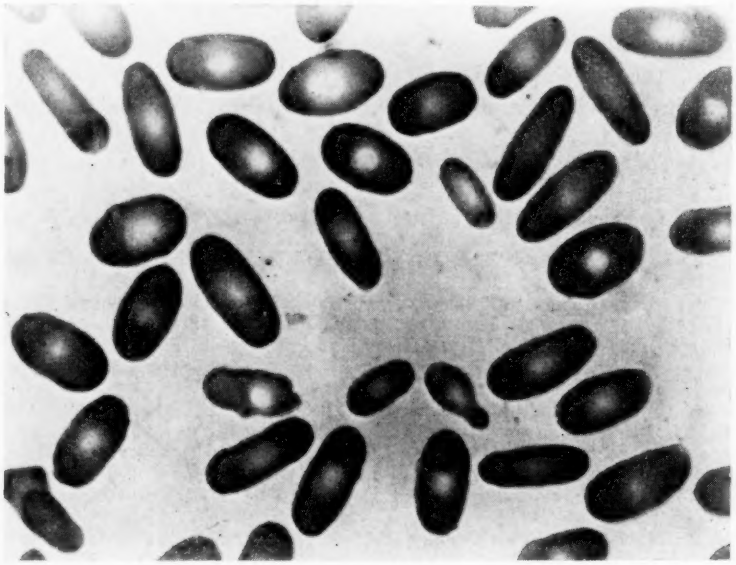

Fig. 1.—Blood film made from specimen obtained by venepuncture. Note the conspicuous elliptical shape of the erythrocytes and the central achromia.

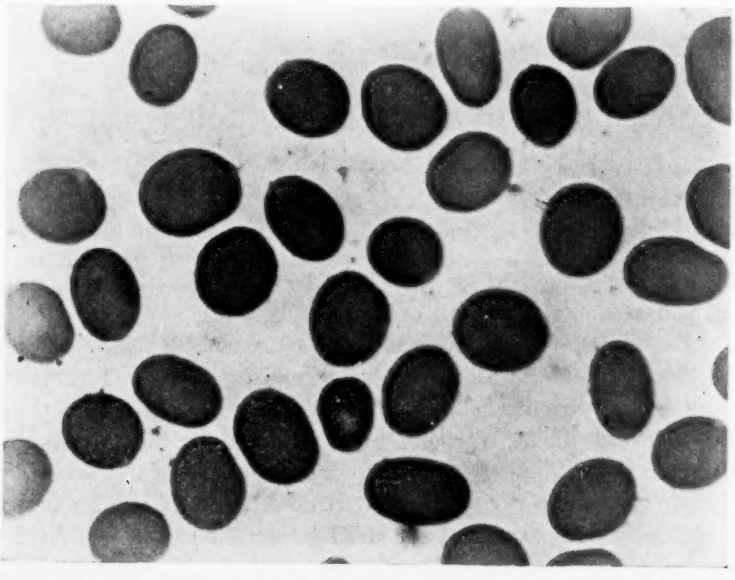

Fig. 2.—Wet sealed preparation. The same blood as shown in Fig. 1 after fifty-six hours. The erythrocytes are either round or distinctly oval. No sickle cells were observed.

that the case presented in this paper is similar to the cases studied by Terry and associates and by Cheney. The fragility test did not reveal any abnormality of the erythrocytes. It is interesting, further, to point out that in film preparations made from the fresh sample of blood obtained by venepuncture, practically all elliptocytes showed a more or less central achromia, but this phenomenon was not observed when the cells were rounded up. No target or sickle cells were observed, but a small number of macrocytes and microcytes and occasionally a poikilocyte were present in the original blood specimen. Their number did not increase in any of the preparations described in this paper. No hemolytic process could be made out at the time the blood sample was obtained. The next of kin of this Negro could not be reached for study purposes.

It is assumed that the case represents an "intermediate type of ovalocytosis." The impression is favored that individuals of this type represent a link between the entities believed by Dameshek to be characterized by erythrocyte thinness, target, elliptical, and oval cells, stippled erythrocytes, hypochromia, and increased resistance to hypotonic sodium chloride solutions.

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PRELIMINARY REPORT ON A RAPID METHOD FOR DIAGNOSING SICKLE-CELL DISEASE

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THE following rapid method for the diagnosis of sickle-cell anemia and sickle-cell trait is based on the prompt occurrence of sickle-cell forms when a drop of sickle-positive blood is mixed with a broth culture of a saline extraction of normally excreted human feces. Soon after mixing a droplet of such blood with a drop of the culture on a slide and covering with a cover slip, the accelerated evolution into crescentic forms can be observed in the microscope. The time required for these changes to develop was found regularly to be less than one hour and in some cases only ten to twenty-five minutes. In three recent cases, the characteristic changes appeared after twenty-five, fifty-five, and

From the Wickersham Hospital, New York, N. Y., and Haemic Research Laboratories, New York, N. Y.
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twenty minutes, respectively. The variations in time depend very probably upon both the sensitivity of the blood and the strength of the unknown factor contained within the broth. Thus the long intervals of six to twenty-four or more hours frequently required by the usual sealed wet preparation method, especially in cases harboring the trait only (Wintrobe[1]), are avoided.

Our method has been studied during the past three years on bloods of twenty-three patients with sickle-cell disease and 108 controls. Twenty-two of those with sickle-cell disease were Negroes and one was a white-appearing Nicaraguan boy born of a Negro mother. Of the controls, fifty-eight were Negroes and fifty were white.

Method.—A cherry-sized quantity of normal human feces (formed, not diarrheic) is mixed with 5 c.c. of normal saline, stirred with a glass rod to a fine suspension, and filtered. Of the resulting filtrate, 0.1 c.c. is transferred into 5 c.c. normal broth and incubated for twenty-four hours at 37° C. The resultant culture fluid is then ready for use. To examine blood for sickling, a drop of this fluid is placed on a slide and a small droplet of the suspected blood gently mixed into it, covered with a cover slip, and examined microscopically.

We have designated this culture as ferment broth for reasons soon to be understood. Following the twenty-four hour period of incubation, it is necessary to keep the ferment-broth either in the refrigerator or at room temperature but not in the incubator. It is also necessary to renew the culture every few days, either by repeating the above procedure or by transplanting into normal broth.

The changes that can be observed in affected blood are attributed by us to a cell-destructive process, terminating in the appearance of sickled red cells. The process may be termed a hemolysis of sickle-cell type. The damaging factor contained in the broth is very probably of an enzymatic nature. It is suggested that this substance might be identical with, or closely similar to, the so-called "blood group property-destroying factor," or "blood group ferment," as elaborated by Schiff and co-workers.[2] The following similarities are enumerated in support of this assumption:

1. The resemblance between our method of production of the ferment broth and theirs
2. The development of the substance by means of incubation
3. The retention of the substance along with the bacteria upon rigid filtration
4. Its easy transferability from one broth to another
5. The action of the substance upon bloods of all the blood groups
6. Its universal presence in the feces of persons of all the blood groups

It may be further stated that:

1. Sickling of red blood cells never failed to appear with our method in any of the bloods in which sickling had occurred in the ordinary sealed wet preparation. Conversely, we found sickling produced in instances in which the ordinary methods failed. The superiority of the method, therefore, appears to reside not only in its acceleration of the phenomenon, but also in the number of cells involved in the change, as well as in the specificity of action.

2. Sickle-cell anemia blood showed a somewhat more rapid appearance of sickling than "trait" blood with our method. This difference in time, however, does not seem to be of too much significance, since it can easily be attributed to

the high sensitivity of such bloods in crisis and to the smaller number of red cells exposed to the diagnostic agent. We consider it an especially fortunate fact that precisely in the "trait," where the underlying condition cannot even be hinted at in a casual examination of the freshly shed blood, are we enabled to follow microscopically the dramatic development through the several well-definable stages toward the final so-called sickle cell. (A description of these transitional stages will be included in our next communication.)

3. The effective agent in the broth containing the saline extract of feces appears in recognizable strength only after its incubation and is then transferable.

4. The pH of the broth culture, in our experience, does not seem to play a prominent role. After twenty-four hours' incubation, the pH of our broth cultures was found to be around 6.7. Thereafter, the acidity increases whereas the effective strength of the culture decreases.

5. Due to the fact that there is no essential difference in the effects produced by our substance on sickle-cell anemia and sickle-cell trait, we are led to consider both conditions as expressions of the same underlying pathology in varying degree and are therefore using the term "sickle-cell disease" for both conditions.

We will present our detailed findings in a more inclusive report to follow shortly.

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ALTERATIONS IN THE CONCENTRATION OF THE BLOOD DURING PREGNANCY

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THIS investigation was undertaken in an effort to determine whether or not there was evidence to substantiate the conviction suggested by the work of Dieckmann and his co-workers, that the alterations observed in the blood of pregnant women are due to a dilution of the blood rather than to an actual systemic loss of the blood constituents. That there is a fall in the concentration of the various blood constituents during pregnancy has long been recognized. Killian and Sherwin,[1] for example, in 1921, and also Caldwell and Lyle,[2] found a definitely lowered concentration of nonprotein nitrogen and urea, while Killian and Sherwin also showed a lowered carbon dioxide combining power during the latter months of gestation but failed to find a lowered uric acid. We[3] established ranges for the carbon dioxide content, the oxygen content and capacity, red cell count, cell volume, and hemoglobin concentration of normal blood of pregnant women in contrast to normal blood of nonpregnant women. One of us (J. W. M.),[4] using improved equipment and methods, found a lowered

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range for uric acid. We[5] showed a fall in both serum calcium and phosphorus. Dieckmann and Wegner[6] demonstrate an increase in both blood and plasma volumes, resulting in a fall in cell volume, hemoglobin, and serum protein concentrations per 100 c.c. of blood. This indicates that there is during pregnancy a dilution of the blood which increases the volume and lowers the concentration.

To secure further information on this, we have determined the specific gravity of the whole blood and of the plasma in a series of women throughout pregnancy, following the same individuals so far as was possible. We also determined the red cell volumes. The specific gravity determinations were made by means of the Eimer and Amend falling drop apparatus, as described by Barbour and Hamilton,[7] with the pipette controller recommended by Guthrie.[8] The details of this method have been given by Drew, Scudder, and Rapps.[9] We modified it to the extent of using heparinized whole blood, which could be taken to the laboratory and carefully checked by repeated readings, rather than using finger blood, with no anticoagulant, in the dispensary or at the bedside. Plasma determinations were made on the same heparinized blood specimens after the cells had been spun down for the cell volume determination. The cell volumes are given in per cent of the cells to the volume of the whole blood used after centrifuging it at high speed until there was no further packing of the cells.

The results, given in Table I, show that from the interval of the twenty-seventh to thirty-second week before delivery to that of the fifth to eighth week, there is a small but uninterrupted fall in the specific gravity of whole blood, of plasma, and cell volume. After a rather abrupt fall between the first two intervals recorded, the changes from one interval to another are too small to have statistical significance. The uninterrupted trend, however, which occurs without exception in all three of the factors measured, is most significant. So also is the fall taken as a whole; for example, the averages of the early interval of twenty-seven to thirty-two weeks before delivery compared to those of the five- to eight-week period give a critical ratio of 5.43 for the whole blood, 5.54 for the plasma, and 4.82 for the cell volumes, showing a very definite statistical significance for all three determinations.

TABLE I

DILUTION OBSERVED IN PREGNANT BLOOD

INTERVAL (WEEKS BEFORE DELIVERY)	SPECIFIC GRAVITY						CELL VOLUME		
	WHOLE BLOOD			PLASMA					
	NO.	MEAN	STANDARD ERROR	NO.	MEAN	STANDARD ERROR	NO.	MEAN	STANDARD ERROR
27 to 32	29	1.0516	0.000477	31	1.0266	0.000255	31	38.0	0.671
21 to 26	58	1.0504	0.000327	58	1.0257	0.000153	57	35.9	0.498
15 to 20	57	1.0493	0.000316	58	1.0255	0.000221	58	35.2	0.305
9 to 14	63	1.0484	0.000312	63	1.0252	0.000193	63	34.7	0.318
5 to 8	43	1.0482	0.000428	45	1.0248	0.000202	44	34.4	0.327
1 to 4	46	1.0495	0.000395	49	1.0251	0.000114	49	36.4	0.404
7 days post partum	57	1.0527	0.000559	57	1.0271	0.000490	57	39.7	0.751

The lowest values, indicating the period of greatest blood dilution, were obtained for the interval of five to eight weeks before delivery. It should be noted, however, that the true minimum might be somewhat outside this arbitrarily selected interval. The rise to the period of one to four weeks before delivery is again small, but noted in all three factors measured, while that from one to four weeks ante partum to seven days post partum is quite marked. The critical ratios for the entire rise are 6.50 for whole blood, 5.03 for plasma, and 6.47 for cell volume, again all being markedly significant.

While one might logically anticipate that this dilution of the blood during pregnancy would be continued until term, the prenatal recovery recorded has also been noted in other determinations. For example, the variation curve of serum calcium and phosphorus reported by us in 1934[5] shows the same tendency. Dieckmann and Wegner[6] also observed that the maximal decrease in hemoglobin concentration occurs between the twenty-sixth and thirty-fifth weeks; that is, five to fourteen weeks before delivery. Dieckmann, however, not only failed to note the prenatal increase during the last four weeks before delivery, but reported the greatest increase in blood volume to be at term. Neither did he observe an immediate post-partum recovery. While we are concerned over this divergence in findings, we feel that we have noted it so distinctly in so many factors that it cannot be ignored. It was apparent not only in the serum calcium and phosphorus, the specific gravity of whole blood, of plasma, and of cell volume, but also in the viscosity of whole blood. Prenatal dispensary admission blood averaged a viscosity of 4.1, with a standard error of 0.049, while seven-day post-partum blood averaged 4.8, with a standard error of 0.215. The critical ratio between these is 3.21, proving that the difference is of definite statistical significance and supporting the observation of immediate post-partum recovery.

We have no explanation for this prenatal recovery, other than that it probably results from a maturity of gestation. There is, however, a most interesting analogy which suggests a possible mechanism for it in the sodium pregnanediol excretion studies made by Bachman, Leekley, and Hirschmann,[10] who find a gradual average increase in the pregnanediol excretion rate from early pregnancy until about two to four weeks before delivery, then a sharp fall until term. Smith, Smith, and Pincus[11] have established a similar prenatal fall in the urinary excretion of estrogens. It would therefore seem possible that hormone regulation of water balance might be responsible both for the blood dilution during gestation and for the slight prenatal concentration during the last few weeks before delivery.

The concentration of the plasma proteins may be obtained from the specific gravity of the plasma according to the formula worked out by Weech,[12] where the plasma protein, in grams per 100 c.c., is equal to the specific gravity of the plasma less 1.0069 times the factor 340.1. We have found this method to be very satisfactory, giving good agreement with other methods of the micro-Kjeldahl type, as, for example, that of Howe.[13] Using this formula we have converted the specific gravity averages of the plasma from Table I to the protein averages for the various intervals recorded, as shown in Table II. This emphasizes the prenatal upward trend and post-partum recovery.

TABLE II

PLASMA PROTEIN DURING PREGNANCY
CALCULATED FROM THE SPECIFIC GRAVITY OF THE PLASMA

INTERVAL (WEEKS BEFORE DELIVERY)	NO.	MEAN SPECIFIC GRAVITY OF THE PLASMA	CALCULATED PROTEIN (GRAMS PER 100 C.C.)
27 to 32	31	1.0266	6.70
21 to 26	58	1.0257	6.40
15 to 20	58	1.0255	6.32
9 to 14	64	1.0252	6.22
5 to 8	45	1.0248	6.09
1 to 4	49	1.0251	6.19
7 days post partum	57	1.0271	6.86

CONCLUSIONS

Evidence has been submitted which indicates that there is a gradual but uninterrupted dilution of the blood from early pregnancy up to a point some five to eight weeks before delivery. From this point on there is a small gradual concentration until delivery, after which the blood quickly returns to non-pregnant levels.

Such a dilution explains the fall in the concentration of the various blood constituents noted by most observers during normal pregnancy.

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LABORATORY METHODS

DEMONSTRATION OF THE CORONARY ARTERIAL SYSTEM WITH NEOPRENE LATEX

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VISUALIZATION of the coronary vascular system has been accomplished largely through the use of injection masses or colored fluids forced into the vessels. Mixtures of barium sulfate and gelatin,[1] various colored coagulants, cellulose, and other substances[2-5] have been utilized successfully for such purposes. In some instances, injection of the system with semisolid material facilitated dissection of large or small vessels; in other cases, corrosion specimens of the coronary system (castings of the vessels) were prepared. By such methods, valuable information has been gained concerning the myocardial vascular bed. More recently Batson[6] and Gamble[7] have reported the use of liquid latex as an injection mass in blood vessels. The substance is readily introduced into the arteries and, when solidified, greatly facilitates their identification. Lieb[8] has used neoprene in a study of the vascular tree of the kidney. Her method of perfusing the renal vessels with neoprene appears to provide a simple technique for study of a vascular system either in corrosion specimens or in microscopic section.

This communication describes a method for the preparation of corrosion specimens of the coronary vascular bed in the dog heart.

METHOD

*Preparation and Infusion of the Coronary Arteries With Neoprene.**—For the injection of the coronary vessels with liquid latex, the general principles of Lieb's technique[8] were followed rather closely. Because of the peculiar anatomic conformity of the coronary arteries, it was necessary to devise a special procedure of injection.

1. Dogs anesthetized with sodium pentobarbital were killed by opening the thorax by sternal cleavage, permitting pulmonary collapse.

The heart, lungs, and trachea were immediately excised en masse. The heart was freed by cutting the pulmonary artery and veins and removing the lungs, trachea, esophagus, and other mediastinal tissue. The pericardium was opened and removed. The heart was then rinsed in tap water at room temperature until the chambers were free of gross blood and clots.

2. Glass cannulae were next inserted into the right and left main coronary arteries. The cannulae were introduced through the coronary ostea within the aortic lumen. One ligature of fine string was utilized to tie the cannula in place in each artery and was secured with a bowknot. Later the bowknots could be untied when the cannulae were withdrawn, and then retied in square knots to ligate the vessels. Therefore, it was necessary to use only one ligature about the main coronary stems, of which the left is very short in the dog heart (detail in Fig. 1).

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*Neoprene latex, Type 571, E. I. du Pont de Nemours and Co.

3. The coronary system was then irrigated with tap water for from four to eight hours. Water was allowed to flow constantly through the heart at a rate of approximately 50 to 75 c.c. per minute.

4. The tubes to the cannulae were clamped, and the heart was placed in the icebox overnight with the cannulae in place and with the tubes and arterial system filled with water. The latter procedure permitted the exclusion of air from the system when neoprene was subsequently infused (see Fig. 1).

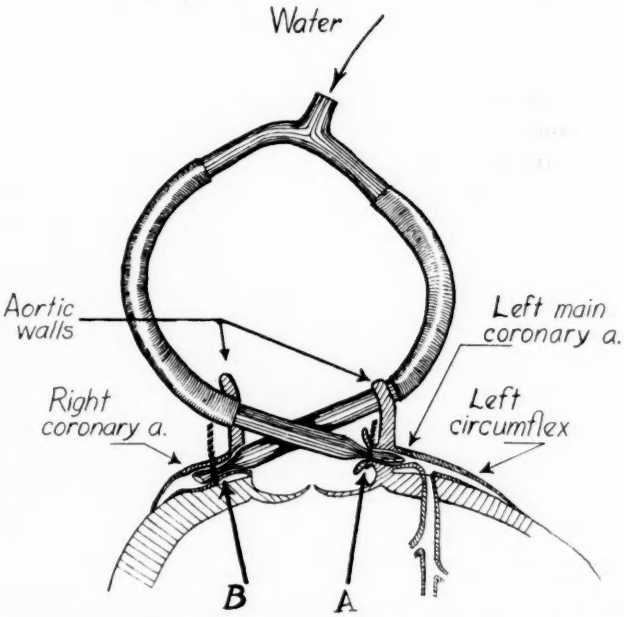


Fig. 1.—Schematic diagram of cannulae in place for irrigation of the heart with water and infusion of the arteries with neoprene latex. Cannulae are inserted into the arteries through the aortic ostea. *A* shows cannula tied into the left main coronary artery; a collar of endothelium about the osteum is dissected up, about which the ligature is secured as shown. This procedure is made necessary by the shortness of the left main coronary. Cannula in the right coronary is secured by a ligature passed around the vessel just without the aortic wall, *B*. Before insertion of the cannulae, the tubes are filled with water and allowed to drip slowly so as to exclude air bubbles.

5. After removal from the icebox, the heart was warmed by being placed in warm running water for thirty minutes or more. The tube leading to the cannulae was then attached to a simple pressure flask containing the neoprene latex at room temperature. Neoprene was allowed to flow into each cannula separately, after careful exclusion of air bubbles from the system. Pressures of 150-170 mm. Hg were applied to the system, and the latex was injected for approximately three minutes into each of the coronary arteries, or until all of the superficial vessels seemed obviously well filled. Following injection, the tubes were again clamped; the bowknots securing the cannulae were untied and were quickly drawn up to ligate the vessels when the cannulae were removed, preventing escape of the neoprene.

6. The heart was transferred to a mixture of 4 per cent acetic acid and 4 per cent formaldehyde for from eighteen to twenty-four hours to fix the tissue and to coagulate the rubber.

Corrosion and Mounting Procedures.—Because of the "basketlike" character of the entire coronary system when the myocardium and other supporting tissues are removed, and the delicacy of the rubber casting of the vessel system, it was necessary to devise a means of supporting the casting in something like its natural conformity. After many trials the following technique was adopted:

1. When the heart had been fixed in the formalin-acetic acid solution, the auricular and ventricular cavities were filled with neoprene. A small, long-stemmed glass funnel was passed through the superior vena cava and one of the orifices of the pulmonary veins into the chambers and neoprene was poured in slowly. The heart was frequently tipped while filling to remove air bubbles. When the chambers were filled, pledgets of cotton soaked in glacial acetic acid were applied to the orifices to prevent escape of the neoprene.

2. The heart was again immersed in the 4 per cent acetic acid-formalin solution for from eighteen to twenty-four hours to permit hardening of the rubber within the cavities.

3. Care was taken to remove all bits of rubber clinging to the outer surface of the heart which might later adhere to the finished specimen.

4. A small glass rod, pointed at one end, was thrust through both ventricular cavities (piercing the lateral ventricular walls), thus impaling the rubber within the heart. The rod pierced the heart below the auriculo-ventricular sulcus to avoid distorting the principal coronary vessels; it was bent into a wide loop so that the organ could be suspended, apex downward, in a jar containing fluid.

5. The suspended heart was immersed in concentrated commercial hydrochloric acid for two days (at room temperature) to allow digestion of all tissues.

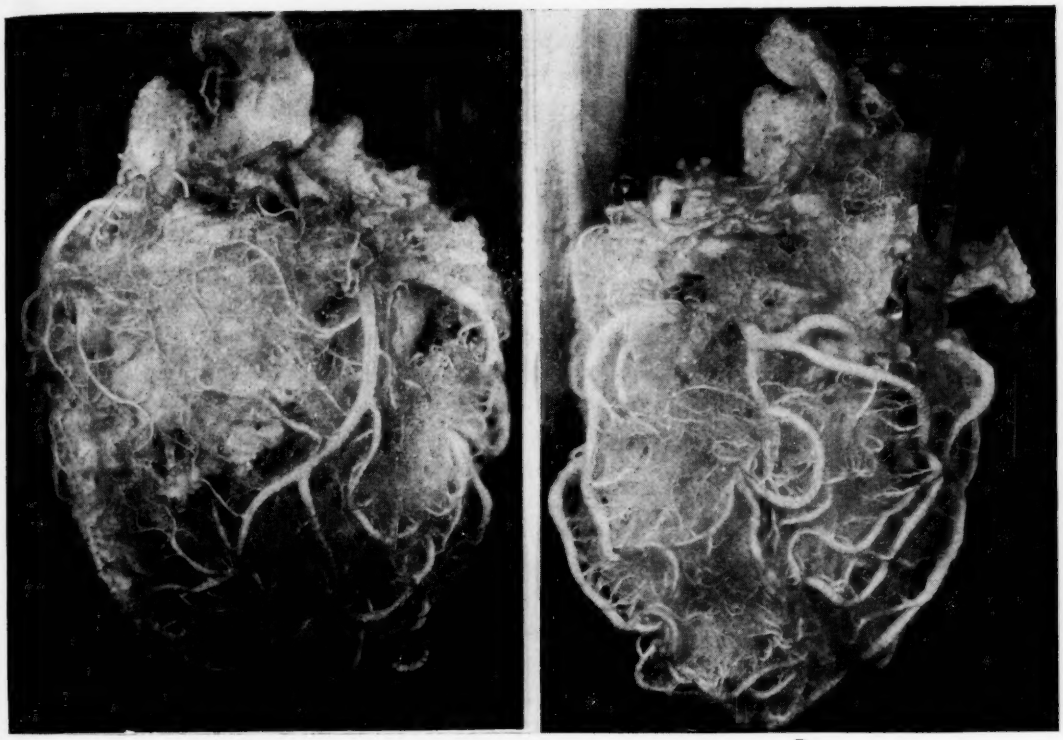
6. The resulting casting of the coronary arterial system is so delicate that care must be taken in removing the acid and digested material. This was accomplished by inserting a small glass tube, connected by rubber tubing to the water faucet, into the acid jar so that its end rested upon the bottom of the jar. Tap water was then allowed to run in slowly for from one to two hours, displacing the acid and digested material. Washing was continued until the casting was free of all particles. The washed specimen was allowed to remain suspended in clear water. Usually glacial acetic acid was added in quantity sufficient to bring its concentration in the bath to about 4 per cent. Such acidification preserves the rubber; the addition of a few crystals of thymol inhibits the growth of mold. If the specimen is to be kept permanently, preservation in Dowicide's solution[8] is advised.

DISCUSSION

The finished specimen, suspended in fluid, is in conformity to the size and shape of the heart. On close examination, the principal coronary arteries and their tributaries are readily identified, and the ramifications of the smaller vessels may be followed into the delicate tracery of the arterioles and to the capillaries which appear as fine wool. The mass of rubber contained in the ventricular cavities is not visible due to the thick, overlying casting of the ventricular arterial system. The auricular walls are so thin that the vessel network permits the rubber contained in the auricular cavities to be seen.

There was some question as to whether the minute vessels, which, in toto, appear as a mass of spun glass, were in reality castings of the capillaries. The individual strands of this mass are probably hardly visible to the unaided eye. Preliminary studies of the perfusion of neoprene into the capillaries, demonstrated by microscopic sections, has shown that the capillary bed is readily injected with neoprene under conditions identical with those occurring in these experiments. It seems to us probable that most of the capillaries were thus filled.

The infusion of neoprene into each of the main coronary arteries for approximately three minutes (under pressures of from 150 to 170 mm. Hg)



A. *B.*

Fig. 2.

Fig. 2.—*A* shows the finished corrosion specimen of the coronary vessel system of the dog heart in anterior view. The specimen is immersed in fluid. The left descending coronary artery and a portion of the left circumflex coronary artery are readily identified. A small section of the right coronary artery is seen on the extreme right border of the casting (to the left in the illustration). Small branches of the arterial system are visible, finally terminating in the smallest vessels.

B shows a left lateral view of the same specimen. Almost the whole of the left descending and left circumflex arteries may be seen; portions of the right coronary artery are visible on the posterior cardiac vascular wall (to the right in the illustration).

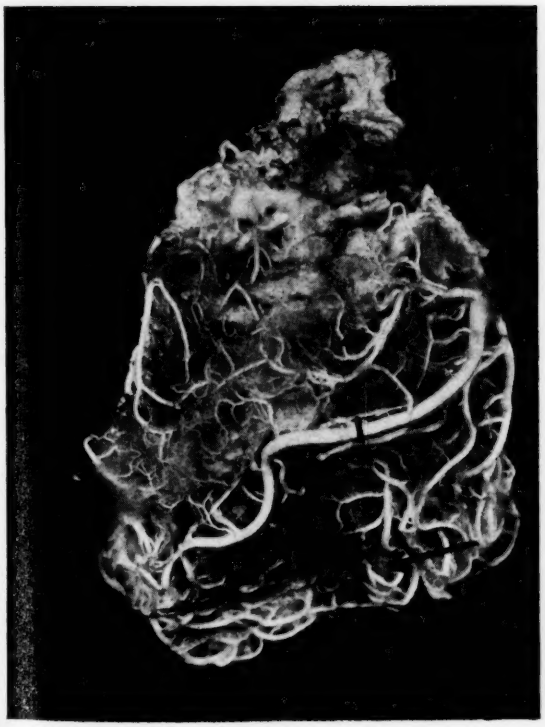


Fig. 3.

Fig. 3.—Corrosion specimen of the coronary system of a dog heart showing an "infarct" in the anterior left ventricular wall. In this heart the left descending coronary artery was ligated at the point indicated by the ink line prior to washing the system with water and infusion with neoprene. The area where vascular filling with neoprene failed to occur is shown by the depressed area indicated by the arrow. Below the point of ligation, the left descending coronary slowly filled with neoprene by retrograde flow from anastomoses with the right coronary vessel.

appeared to be adequate for reasonably complete filling of the arterial system. When the rubber mixture was permitted to flow into the arteries for longer periods of time, it frequently passed the capillary bed to fill the large cardiac veins. Although demonstration of the cardiac veins filled with latex may be useful in certain types of observation, it was not desired in these experiments. Venous filling was thus prevented by limiting the time of inflow of rubber mixture.

We have found it necessary to keep the specimen suspended in fluid. When removed from the bath, the entire casting collapses into a small, amorphous mass. When replaced in water, the strands of the casting will again separate and float freely so that the larger vessels may be identified, but the most minute strands do not separate from each other and the capillary portions of the exhibit are permanently lost. Therefore, unless a portion of the specimen is to be cut away for special examination, the rubber casting is best kept constantly immersed.

A corrosion specimen of the coronary system may be prepared without attempting to support it by filling the cardiac chambers with latex. The unsupported casting may then be cut longitudinally in any place and floated in water to exhibit the course of blood vessels in plane surface.

Such preparations are useful in demonstrating the extent of the normal coronary vascular system. However, it appears to us that the method may attain greatest usefulness in the study of anastomoses between the principal coronary vessels, and the extent of collateral circulation following coronary occlusion. Our preliminary studies indicate a high degree of anastomosis between the main coronary vessels; the size of infarcts, following the coronary closure, may be dependent upon the presence or absence of such anastomoses (see Fig. 3).

SUMMARY

A method is described whereby neoprene latex is infused into the coronary vessels of the dog heart. Corrosion specimens, employing concentrated hydrochloric acid, may be made which appear to preserve all details of the coronary arterial system lumina including the capillaries. The cardiac chambers may be filled with liquid latex and the whole organ suspended by a glass hook to support the vascular casting when the myocardium is digested away. The finished specimen is kept immersed in fluid so that the minute strands are separated and details of the coronary vascular system may be seen.

The authors are indebted to Dr. Robert A. Moore, Department of Pathology, for many helpful comments and for the suggestion that neoprene be used in these experiments. The Rubber Laboratories of E. I. du Pont de Nemours and Co. kindly furnished the neoprene latex used in these experiments.

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A RAPID PRESUMPTIVE COMPLEMENT FIXATION TEST FOR SYPHILIS

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THE rapid presumptive complement fixation test to be described is useful in weeding out possible cases of syphilis in physical fitness groups, in pre-transfusion donor testing, and in similar situations where any rapid presumptive test is needed. It does not in any sense replace the standard Kolmer, Kline, Hinton, Eagle, or Kahn tests and should be checked by one or more of the standard tests as soon as practicable. The reaction depends on the native complement and the native hemolysin against guinea pig erythrocytes present in the examined blood. Either or both of these may occasionally be weak or absent, in which case the test yields a false positive result. However, since a diagnosis is not made on this method of testing, an occasional false positive reaction is inconsequential. The test is simple and requires very little equipment not usually found at medical stations. (An orange crate will house four pigs, a month's supply, if an animal is sacrificed at each bleeding.)

Method.—

1. Transfer 1 c.c. of freshly drawn venous blood into a small tube containing 0.5 c.c. of Kolmer antigen ($\frac{1}{75}$*) and shake vigorously.

2. Place the tubes in a water bath at 37 to 39° C. for one-half to one hour.

3. Remove tubes from the water bath and cut the clot loose if it is adherent to the tube wall.

4. Centrifuge the tubes until the supernatant stratum is free from erythrocytes.

5. Fill a white blood cell-counting pipette to the bulb with the supernatant, erythrocyte free, serum-antigen mixture, continue filling the pipette to the "11" mark with 1 per cent washed guinea pig erythrocytes, and mix.

6. Incubate under desk lamp at 37 to 38° C. for fifteen minutes.

A hemolytic control tube is not routinely used since all the tests showing incomplete hemolysis are checked routinely by standard methods, regardless of the cause of inhibition. If desired, a control may be set up by substituting 0.5 c.c. of 0.85 per cent saline solution in step 1 for the 0.5 c.c. of antigen mixture. This will show if the natural complement-hemolysin content is adequate. Equipment required for performance of the test is illustrated in Figs. 1 and 2.

A negative test shows complete hemolysis of the guinea pig cells in the pipette bulb; the white bead appears sharp in outline. A strongly positive test shows no hemolysis, the bulb contents being opaque and the agitating bead indistinct. After the cells sediment to the bottom of the bulb, the supernatant fluid is water clear. Weakly positive tests show partial but not complete hemolysis. After standing some time, the sedimented cells have a tendency to agglutinate. Rabbit erythrocytes are not suitable because of the lower natural hemolysin content of human serum against them and a much greater tendency

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*We have tried several Kolmer antigens, some standard stock antigens furnished by the U. S. Navy, and some of our own extraction. The stock antigen is of such strength that if employed in the standard Kolmer test it is used in .5 c.c. quantities of a 1/500 dilution. It is mixed by placing .2 c.c. of stock antigen into a clean, dry, Pyrex, four-ounce, small-neck nursing bottle and adding drop by drop 15 c.c. of isotonic saline with vigorous shaking.

to agglutinate. Washed 1 per cent guinea pig cells (three washings) suspended in 0.85 per cent saline kept at 8° C. are usable for a week. We have checked this test against a standard Kolmer, a standard Kahn, and clinical history and diagnosis. A few false positive reactions are encountered, but we have not missed a full 4 plus reaction, and this test compares favorably with other tests in weakly positive cases having a lower reagin content. There is at least not more conflict than we find between the Kolmer and Kahn tests as done routinely. Since there is so little processing of the few reagents, technical errors are minimal.

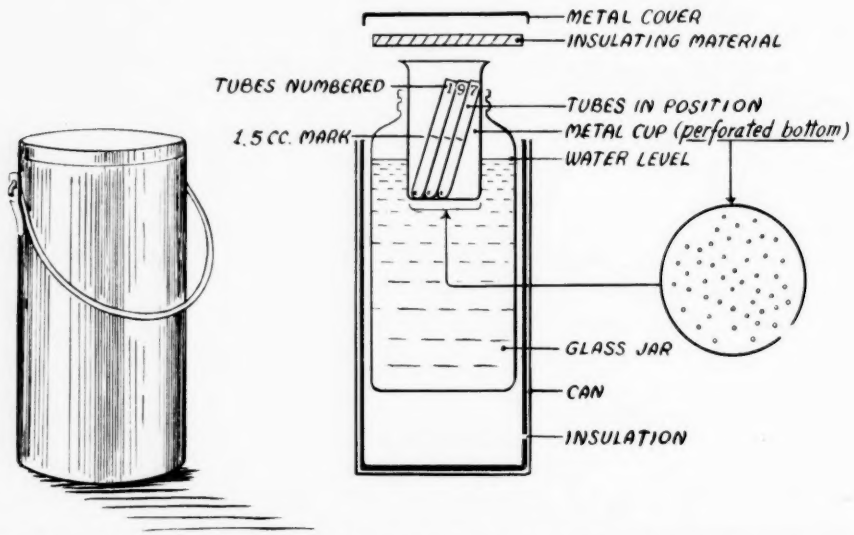

Fig. 1.—A portable, efficient water bath can be made with a fruit jar and a perforated can to fit in the top and with some sort of cloth or paper insulation.

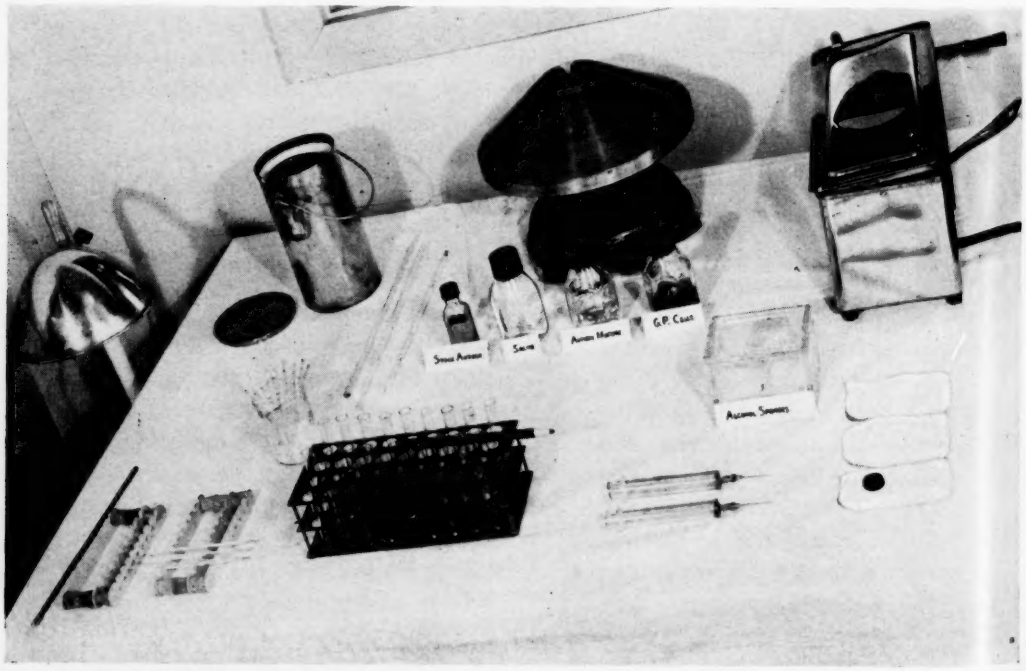

Fig. 2.—The setup conveniently placed on a 32 by 46-inch typewriter desk top with room to spare. The larger tubes in the test tube rack are for the purpose of collecting additional blood for follow-up serology or other tests.

We have completed 724 tests of blood obtained from patients in the skin, surgical, and medical wards with various diagnoses, including many tropical diseases. The patients were almost all men between 18 and 45 years of age—white, Negro, and Oriental. Each presumptive test was controlled by a standard Kahn and a quantitative Kolmer test. In this group were 124 cases in which there was a strongly positive reaction in all three tests. There have been four strongly positive presumptive tests which were negative by the standard tests. One of these was in a patient with a skin lesion diagnosed papulo syphilodum on admission and one was in a patient with a diagnosis of hookworm, who gave a history strongly suggestive of yaws. In the other two cases the patients showed no clinical evidence of syphilis and gave negative histories. Emulsions from lymph glands of these patients injected into testes of rabbits produced no lesions (still under observation). Two early cases were positive by the presumptive test eight and ten days, respectively, before the standard tests were positive. Six cases were not as strongly positive as the standard tests. Eighteen patients showed doubtful reactions, which were negative by the standard tests.

Acknowledgment is made of technical assistance of Nathan T. Hodges, Ph.M. 1/c, U.S.N.R., who independently conducted the control Kolmer and Kahn tests and in other ways assisted in the setup.

THE USE OF PLASTICS TO REPLACE COVER GLASSES IN MICROSCOPE SLIDE PREPARATIONS

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THIS paper deals with a method of applying plastics to microscope slide preparations, the main purpose being to eliminate the routine use of cover glasses. Persons familiar with these preparations realize the tedium of cleaning and placing cover glasses. This fastidious, slow, and therefore, relatively expensive procedure has often been a source of annoyance to me, and has led to the present study as a means of eliminating it. The object is to develop a multiple slide procedure which can be carried on in a shorter time and at a decreased cost.

MATERIALS

The materials selected were plastics which were especially recommended for coating purposes.

The desired properties were: (1) strong adhesion to glass and tissue, (2) maximum optical transparency, (3) resistence to abrasion and to common solvents, (4) slow or negligible deterioration by light and chemicals, and (5) ease of application.

Several types of plastics were tried in order to find suitable ones. The nitrocellulose plastics offered some desirable properties, but these were offset by an observed poor resistance to ultraviolet radiation. Several alkyd resins were found unsatisfactory. The two plastics which appeared most satisfactory in this preliminary work were Acryloid B-72* and Vinylseal.†

From the Department of Pathology, West Virginia University Medical School.
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*Obtained from The Resinous Products & Chemical Co., Philadelphia, Pa.
†Obtained from The Bakelite Corporation, New York, N. Y.

Acryloid B-72 is an acrylic resin obtained from the manufacturer in 40 per cent solution of toluol. Its chief disadvantage is a pronounced solubility in xylol and other common solvents, although its coating and optical properties were found to be excellent. This plastic was superior to the others used in this experiment, and its resistance to ultraviolet light is enormous. Solutions made thinner with toluol can be used for blood, bacterial, and exudate smears.

Vinylseal is a vinyl acetate resin in methyl acetate (or other solvents). This is rather resistant to xylol, but it does not have the coating qualities of the Acryloid B-72, and its use requires much greater care. Further work with vinyl resins should reveal definite possibilities. At the present time I do not use vinyl resins.

It should be stated that an exhaustive study of a greater number of plastic substances should bring to light materials which have even more desirable properties than have those upon which this report is based. Rather than to make a detailed study of all of the available plastics, it was considered more desirable to submit this preliminary report in order to stimulate further investigation.

METHOD

The technique is applicable to one or any number of slides. A proper multiple slide holder, such as the Fisher-Brubach holder, is used if several slides are to be prepared.* After the preparation has been stained and passed through the alcohols and xylol, it is dipped slowly into the plastic solution and allowed to remain for about thirty seconds. It is then slowly lifted out, supported in a rack over the solution at an angle so that the excess plastic drains off. This procedure is usually repeated once, and when the plastic ceases to flow, the holder is hung on a rack to permit drying of the slides. The number of applications of this plastic depends upon the thickness desired, and this in turn depends upon the thickness of the section.

After complete drying, the slides may be examined under the microscope with little risk of damage. However, if the slides are placed in an ordinary drying oven and baked at 140° C. for one hour, the plastic, on cooling, will be very hard and much more resistant to abrasion. This procedure must be followed if the slides are to be filed.

APPLICATION

The chief purpose of this technique is its application to fixed tissue preparations in order to eliminate cover glasses for routine use. It may also be applied by using thinner solutions of plastic to various preparations such as blood, bacterial, and exudate films for the purpose of providing protection and making a better-appearing slide, although obviously this is unnecessary. I have, however, used it in making preparations of parasite ova, cysts, and the like, and find it extremely simple and superior to many other methods. In this case the concentrated fecal material containing ova or cysts is smeared on the slide, allowed to dry, and dipped into the plastic solution. An alternate method consists of allowing the plastic to flow over the film and to dry. This method also probably can be used in replacing the cumbersome Canadian balsam technique in making Golgi preparations for nerve tissues.

There are certain possible disadvantages, the elimination of which will require additional work. In regard to Acryloid B-72 preparations, one may be

*A special tray, slide holder, and rack are being devised for this technique.

of importance. This plastic is fairly soluble in xylol and certain other solvents, and care should be taken to see that such solvents do not come into contact with the slide. However, if this should occur, a new surface can readily be applied. Immersion oil has no adverse effect; in the event that it should dry on the slide, it may readily be removed by adding more liquid oil and wiping.

The advantages are obvious. This procedure would completely eliminate cover glasses in routine preparations, decrease cost, increase speed of preparation, and simplify multiple preparations, such as serial sections of embryos, brain preparations, and the like. Labels, if applied to plastic with a solvent, become extremely adherent. If valued slides were to fade in time, they could be restained and resurfaced with ease. In addition, application is almost universal to all types of slide preparations.

Slides kept so far for more than six months look exactly as they did the day they were prepared, even though they have been given rough treatment.

SUMMARY

This paper presents a new technique; namely, the application of plastics to replace routine use of cover glasses in microscope slide preparations.

The author wishes to express his sincere and deep appreciation for the invaluable aid rendered by Dr. Howard Perry Simons, Associate Professor of Chemical Engineering, West Virginia University. He not only helped with his knowledge of plastics, but also corrected the original manuscript.

The Resinous products & Chemical Company and the Bakelite Corporation kindly and generously donated some of the plastics which made this work possible. To these companies the author is greatly indebted.

BOOK REVIEWS

The Specificity of Serological Reactions. By *Karl Landsteiner.** Revised edition, 1945, Harvard University Press. Cambridge, Mass., Price $5.00. Cloth with 310 pages.

The importance of Landsteiner's book appears in its proper light when one reflects that nearly all the fundamental problems of biology are concerned either with the phenomenon of specificity or that of growth. It is clear from this book that its author was attentive all his life to the basic problems of biological specificity, and it is this preoccupation, even more than Landsteiner's contributions to the various branches of immunology, which recommends the book to new readers today. To readers of the earlier (1936) version, who include all immunologists, the revised edition will need no recommendation.

The revision of an authoritative work in any field provides an opportunity to see clearly the development of the subject. The field of immunochemistry is remarkable both for the newness and, in certain respects, the completeness of its present stage of development. The newness must be urged especially for the younger people who are likely to take too much for granted Landsteiner's proof of the chemical basis of serological specificity. For them Landsteiner (page 156) calls attention to the state of affairs that existed as recently as 1917, when Morgenroth justly wrote of "serology as a field to which there leads no bridge from chemistry." To the extent that natural immunology is largely a branch of protein chemistry, this state of affairs might be little changed today except for the successful use of artificial models introduced by Landsteiner and his colleagues in the very year (1917) of Morgenroth's comment.

By 1936, when the first American edition of Landsteiner's book appeared, the situation was very different, and Landsteiner wrote (page 136), "These results [correlating serological reactions and chemical structure] have provided prerequisite data for the theoretical treatment of serological specificity, and the path of further advance along this line can be foreseen." The correctness of this estimate appears from the present edition, complete according to its foreword at the time of Landsteiner's death in 1943, which records the active progress of research during the intervening years but does not mention any essentially new methods or principles.

The new edition follows closely the outline of the old but is considerably expanded both by the inclusion of new material and the introduction of new topics serving, in the words of the author's preface, "to give an outline of the field of immunochemistry." The greatest value of the work remains the same as before; that of providing the only text available on the subject which is either clear enough to be readable by anyone with an elementary background in chemistry and biology or comprehensive enough to meet the needs of the specialist. This is primarily true of course with respect to that portion of the field of immunochemistry to which Landsteiner devoted such prodigious labor: the relation between chemical structure and serological specificity. Not readily available elsewhere, also, is the material collected in the several chapters

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devoted to the natural history of antigens, in which approximately equal attention is paid to the chemistry, and to the genetic and phylogenetic relationships, of animal and bacterial antigens.

Other topics, such as physico-chemical and theoretical considerations of the immune reactions and the origin of antibodies, are treated in a frankly synoptic and uncritical manner, as is appropriate to the nature of the work, which is first of all that of a great experimenter. These topics are nevertheless faithfully noted in the text and are included in the generally complete bibliography.

The attention of the physician may be called especially to the chapter entitled "Hypersensitivity to Substances of Simple Composition" (pages 197-207), which includes a review of the experimental demonstration, principally by Landsteiner and Jacobs, and Landsteiner and Chase, of the immunologic mechanism responsible for at least one kind of contact dermatitis.

The book closes with a brief but characteristically lucid chapter by Linus Pauling on the structure of molecules and the nature of the known intermolecular forces. This is concluded by a very clear picture of the way in which large molecules may be understood to combine both firmly and specifically as a result of the coordination of many weak interatomic bonds operating in the area of contact between complementary surfaces.

A. D. Hershey.

The Practice of Medicine. By *Jonathan Campbell Meakins,* M.D., LL.D., Brigadier, Deputy Director General of Medical Services, Royal Canadian Army Medical Corps; Professor of Medicine and Director of the Department of Medicine, McGill University; Physician-in-Chief, Royal Victoria Hospital, Montreal; Formerly Professor of Therapeutics and Clinical Medicine, University of Edinburgh; Fellow of the Royal Society of Physicians, London; Fellow of the Royal College of Physicians, Edinburgh; Honorary Fellow of the Royal College of Surgeons, Edinburgh; Fellow of the Royal College of Physicians, Canada; Fellow of the American College of Physicians; Honorary Fellow of the Royal Society of Medicine. Fourth edition. The C. V. Mosby Company, St. Louis, Mo., Price $10.00. Cloth with 1,444 pages and 517 illustrations including 48 in color.

Mass Miniature Radiography of Civilians for the Detection of Pulmonary Tuberculosis. By *Kathleen C. Clark,* M.B.E., F.S.R., Principal, Radiographic Technical and Demonstration Department, Ilford Limited; *P. D'Arcy Hart,* M.D., F.R.C.P., Member of the Scientific Staff, Medical Research Council; *Peter Kerley,* M.D., F.R.C.P., F.F.R., D.M.R.E., physician to X-Ray Department, Westminster Hospital and Royal Chest Hospital; and *Brian C. Thompson,* M.D., Tuberculosis Physician, Middlesex County Council. Privy Council, British Medical Research Council, Special Report Series No. 251. London: His Majesty's Stationery Office, 1945. Price 3s. Od. net. Paper with 135 pages.

1944 Year Book of Dermatology and Syphilology. Edited by *Marion B. Sulzberger,* M.D., Commander, M.C., U.S.N.R.; Assistant Clinical Professor of Dermatology and Syphilology, New York Post-Graduate Medical School of Columbia University; Member of the American Dermatological Association, Inc.; Assistant Editor *Rudolf L. Baer,* M.D., Assistant Attending

Physician, Skin and Cancer Unit, New York Post-Graduate Hospital, Columbia University; Diplomate, American Board of Dermatology and Syphilology. Year Book Publishers, Inc., Chicago, Ill. Price $3.00. Cloth with 544 pages.

1944 Year Book of Industrial and Orthopedic Surgery. By *Charles F. Painter*, M.D., Orthopedic Surgeon to the Massachusetts Women's Hospital and Beth Israel Hospital, Boston. Year Book Publishers, Inc., Chicago, Ill. Price $3.00. Cloth with 432 pages.

Casualty Work for Advanced First-Aid Students. By *A. W. MacQuarrie*, M.B., Ch.B. (Edin.), Admiralty Surgeon and Agent Civil Defence Medical Officer; Major and Battalion Medical Officer, Home Guard. E. and S. Livingstone, Ltd., 16-17 Teviot Place; imported by the Peter Reilly Co., Philadelphia, Pa. Price $1.80. Cloth with 231 pages.

ANNOUNCEMENT

In compliance with the directives imposed by the War Production Board limiting the amount of paper consumed in the production of this JOURNAL, the publishers find it necessary to change the format. As soon as these restrictions are lifted the original format will be restored. Even though the number of pages has been reduced, the actual content of the JOURNAL has not been decreased to any appreciable extent.